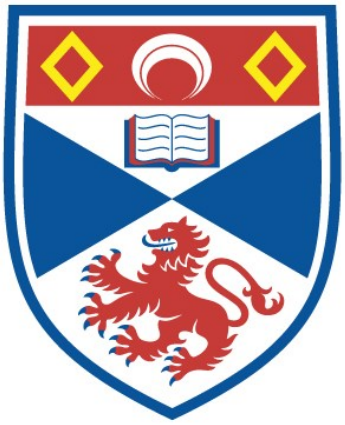
THE DEVELOPMENT AND USE OF ANTIGEN-ANTIBODY-LTB (AG-MAB-LTB) COMPLEXES AS IMMUNOGENS

Elizabeth Allison Green

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1995

Full metadata for this item is available in
St Andrews Research Repository
at:
http://research-repository.st-andrews.ac.uk/

Please use this identifier to cite or link to this item:
http://hdl.handle.net/10023/13984

This item is protected by original copyright

THE DEVELOPMENT AND USE OF ANTIGEN-ANTIBODY-LTB (Ag-MAb-LTB) COMPLEXES AS IMMUNOGENS.

By Elizabeth Allison Green, B.Sc.
Division of Cell and Molecular Biology
University of St. Andrews

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Science at the University of St. Andrews, February 1995.

BIBLIOTHECA UNIVERSITATIS ANDREANÆ

ProQuest Number: 10166324

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10166324

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

TL
B712

DECLARATION

(a) I, Elizabeth Allison Green, hereby certify that this thesis has been composed by myself, that it is a record of my work and that it has not been accepted in partial or complete fulfillment of any other degree or professional qualification.

Signed..... Date.....8/2/95.....

(b) I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 1st October 1990 and as a candidate for the degree of Ph.D. on 1st October 1991.

Signed..... Date.....8/2/95.....

(c) I hereby certify that the candidate has fulfilled the conditions of the Resolutions appropriate to the ~~Degree of~~ Ph.D.

Signature of Supervisor..... Date.....8/2/95.....

(d) In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any *bona fide* library or research worker.

ACKNOWLEDGEMENTS

I would like to extend the warmest of thanks to my supervisor, Dr. Rick Randall, for his total support, encouragement and informative discussions throughout this thesis. I would also like to express my appreciation to Professor William Russell, Dr. Catherine Botting and Dr. Paul Szawlowski for their enormous moral support during the writing of this thesis. I wish to thank Dr. T.R. Hirst for providing me with the plasmid pTRH101R and *Vibrio sp.* 60, as well as Dr. G. Kemp for his help in using the FPLC system. A special thank you goes to Dr. A Mowat at Glasgow university for providing the cytokine-ELISA assays, the technicians in our department and Bill Blyth for his photographic skills. I am also indebit to my family and friends for their unwavering belief in me. And to the people of lab.28, both past and present, thanks for all the laughs through the years, and providing me with so many happy memories of my time in St. Andrews.

Finally, I am grateful to the Medical Research Council for providing financial support during the course of this study.

This thesis is dedicated to the memory of my Grandfather.

ABSTRACT

In the course of this work a novel strategy has been developed for linking the adjuvant *Escherichia coli* heat-labile enterotoxin subunit B (LTB) to Simian Immunodeficiency Virus (SIV) proteins via an antibody bridge and the systemic and mucosal immunogenicity of such SIV-MAb-LTB complexes have been investigated.

A short peptide tag, termed Pk, was joined to the 3'-end of the gene coding for LTB and expression studies revealed that the gene product, LTB-Pk, could be efficiently synthesised and secreted from non-pathogenic *Vibrio sp.*60. Analysis of the functional properties of LTB-Pk demonstrated that LTB-Pk , like native LTB, was a heat-labile oligomer, that could bind to the glycolipid GM1-ganglioside and was immunogenic *in vivo*. In attempts to purify LTB-Pk for immunisation studies, both hydrophobic and ion-exchange chromatography schedules were analysed, the latter procedure being more efficient. Strategies were developed for joining LTB-Pk to one arm of an anti-Pk MAb, (MAb SV5-P-k) and Pk-linked SIV proteins to the other arm, and such SIV-MAb-LTB complexes bound to GM1-ganglioside *in vitro*. Systemic immunisation studies suggested that SIV-MAb-LTB complexes, using recombinant p17 as the target antigen, promoted both humoral and cell-mediated immunity to the recombinant p17. In addition, it was later shown that conjugation of LTB-Pk to recombinant SIV proteins via an antibody bridge, resulted in a more efficent presentation of the recombinant SIV protein to the immune system , than co-administration of LTB-Pk with the recombinant SIV protein. However, intranasal administration of p17-MAb-LTB complexes did not induce immunity to recombinant p17. Subsequently it was shown that the recombinant p17 was highly susceptible to mucosal degradation, suggesting the poor mucosal immunogenicity of p17-MAb-LTB complexes may be related to the instability of recombinant p17 in the mucosal environment. Further investigations into the stability of other recombinant SIV proteins in the mucosa, revealed that recombinant p27 was more resilient to mucosal degradation. p27-MAb-LTB complexes were constructed and initial intranasal immunisation studies revealed that both systemic and cell-mediated immunity to recombinant p27, could be induced following intranasal administration.

Furthermore, mucosal immunity to recombinant p27 was evident in the lungs of vaccinated mice, with anti-recombinant p27 IgG-secreting cells predominating.

ABBREVIATIONS

NUCLEIC ACIDS

DNA	2' deoxyribonucleic acid
RNA	ribonucleic acid
A	adenine (base in DNA/RNA)
G	guanine (base in DNA/RNA)
C	cytosine (base in DNA/RNA)
T	thymine (base in DNA)
U	uracil (base in RNA)
ATP	adenosine 5' triphosphate
GTP	guanosine 5' triphosphate
cAMP	cyclic 3', 5' adenosine monophosphate
mRNA	messenger RNA

AMINO ACIDS

A	Ala	alanine	M	Met	methionine
C	Cys	cysteine	N	Asn	asparagine
D	Asp	aspartate	P	Pro	proline
E	Glu	glutamate	Q	Gln	glutamine
F	Phe	phenylalanine	R	Arg	arginine
G	Gly	glycine	S	Ser	serine
H	His	histidine	T	Thr	threonine
I	Ile	isoleucine	V	Val	valine
K	Lys	lysine	W	Trp	tryptophan
L	Leu	leucine	Y	Tyr	tyrosine

PHYSICAL UNITS

°C	temperature in degrees Celsius
g	gram mass or centrifugal force
mg	milli gram (10^{-3} g)
µg	micro gram (10^{-6} g)
ng	nano gram (10^{-9} g)
l	litre volume
ml	milli litre
µl	micro litre

Ci	Curie (measure of radioactivity = 3.7x 10^{10} disintegrations/second)
μCi	micro Curie
M	molar concentration
mM	milli molar
μM	micro molar
kD	kilodalton
kb	kilobase (pairs)
pH	$-\log_{10}[H^+]$
V	volts
mA	milli amperes
U	units of enzyme activity
in^2	inches squared
lb	pounds
cpm	counts per minute
Å	angstrom (10^{-10})

CHEMICALS AND REAGENTS

FCS	Foetal Calf Serum
EDTA	ethylenediaminetera-acetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetra-acetic acid
NP-40	nonidet P-40
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylethylenediamine
DATD	N, N-diallyltartardiamide
FITC	fluoroscein isothiocyanate
PBS	phosphate buffered saline
Tris-HCl	tris-hydroxymethyl-aminomethane, pH adjusted with HCl
TE	tris EDTA
MOPS	3-(N-morpholino) propane sulphonic acid
^{125}I	radioisotope iodine-125
^{3}H	radioisotope hydrogen-3
BCIP	5-bromo-4-chloro-3-indoyl phosphate
AMP	2-amino-2-methyl-1-propanol
IPTG	isopropyl-β-*D*-thiogalactopyranoside
TBE	Tris-borate-EDTA buffer

Triton	t-Octyphenyloxpolyethoxyethanol

VIRUSES

SV5	simian virus type 5
RSV	respiratory symcytial virus
LCMV	lymphocytic choriomeningits virus
HIV#	human immunodeficiency virus
FMDV	foot and mouth disease virus
SIV	simian immunodeficiency virus
HSV-2	herpes simplex virus-2

MISCELLANEOUS

ADCC	antibody -dependent cell-mediated cytotoxicity
APC	antigen presenting cell
AIDS	acquired immunodeficiency virus
ASC	antibody secreting cell
Ab	antibody
B cells	B lymphocytes
BSA	bovine serum albumin
BCG	bovine bacillus Calmette-Guerin
CD#	cluster designation
CFTR	cysitic fibrosis transmembrane receptor
CTL	cytotoxic T cell
CMI	cell-mediated immunity
C-terminus	carboxyl terminus
CT	cholera toxin
CTB	cholera toxin subunit B
D_H	diversity gene of immunoglobulin heavy chains
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
env	envelope
ER	endoplasmic reticulum
e.g.	for example
E.coli	*Escherichia coli*

ECL	enhanced chemiluminscence
Fab	antigen-binding fragment of antibody
Fig.	figure
FPLC	fast protein liquid chromatography
Fc	crystalisable fragment of antibodies
FcR	receptor for the Fc region of antibodies
FCA	Freund's complete adjuvant
FDC	follicular dendritic cell
gag	group-specific antigen
GM1	GM1-monosialoganglioside
gp	glycoprotein
GST	glutathione-*S*-transferase
H-2	designation for the murine MHC locus; chromosome 17
H	heavy chain of antibodies
HRP	horseradish-peroxidase
HBcAg	Hepatitis B virus core antigen
HBsAg	Hepatitis B virus surface antigen
Ig#	immunoglobulin
IL-#	interleukin
IFN-γ	gamma interferon
J_H	joining gene of immunoglobulin heavy chains
L-broth	Luria broth
L-agar	Luria agar
L	light chain of antibodies
ISCOMS	immunostimulatory complexes
LT	*E.coli* heat-labile enterotoxin
LTB	*E.coli* heat-labile enterotoxin subunit B
MAb	monoclonal antibody
MHC	major histocompatibility complex
MDP	*N*-acetylmuramyl-L-analanyl-D-isoglutamine
MAPS	multiple antigen peptide system

O.D.	optical density
NTA	nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
Pr.A	protein A component of *Staphylococcus aureus*
PHA	phytohaemagglutinin
rIL-2	recombinant interleukin-2
SMAA	solid matrix-antibody-antigen complex
St. aureus	*Staphylococcus aureus*
T cells	T lymphocytes
TcR	T cell receptor
Ty:VLP	Ty: virus-like particle
T_H#	T helper cell
TNF#	tumour necrosis factor
t-MDP	threonyl-MDP
U.V.	ultra violet
V_L	variable region of antibody light chains
V_H	variable regions of antibody heavy chains
v/v	volume per total volume ratio
WB	western blotting
w/v	weight per total volume ratio
<	less than
>	greater than
%	percent

CONTENTS

INTRODUCTION 1

A. ADAPTIVE IMMUNE RESPONSE TO ACUTE VIRAL INFECTIONS 2

A.1. Humoral Immunity to Viral Infections 2

A.1.1. Classification of antibodies 3

A.1.2. Activation of the B cell 3

A.1.3. Generation of diversity of the B cell receptor 4

A.1.4. Determinants recognised by the B cell receptor 5

A.1.5. Evasion of the humoral response 6

A.2. Cell-Mediated Immunity to Viral Infections 7

A.2.1. Antigen presenting cells (APCs) 8

A.2.2. The Major Histocompatibility Complex (MHC) 9

A.2.3. The T cell receptor (TcR) 10

A.2.4. Pathways of processing and presentation of antigens 12

A.2.5. Immune regulation; influence of the immune response genes 16

A.2.6. Viral escape of cell-mediated immune responses 17

A.2.7. Cellular immune responses to viruses and implications for vaccine design 18

A.3. Deleterious versus Protective Responses 18

A.4. Long-Lived Immunity 19

A.5. Mucosal Immunity to Viruses 20

A.5.1. Evidence of a common mucosal immune system 20

A.5.2. Antigen uptake at the mucosal surfaces 21

A.5.3. Humoral immunity at the mucosal surfaces 22

A.5.4. Epitopes recognised by B cells at mucosal surfaces 24

A.5.6. Cell-mediated immunity at the mucosal surfaces 24

A.5.7. Intraepithelial lymphocytes 25

B. VACCINATION 26

B.1. Live, Attenuated Viral Vaccines 26

B.2. Whole, Killed Virus Vaccines 27

B.3. Subunit Vaccines; A Reductive Approach to Vaccination 28

B.3.1. Peptides 29

B.3.2. Purified, recombinant viral proteins 30

B.4. Novel Strategies for Potentiating the Immune Response to Subunit Vaccines 31

B.4.1. Adjuvants 31

B.4.2. Novel carrier systems 34

B.4.2.1. Live, recombinant viral vectors 35

B.4.2.2. Live, recombinant bacterial vectors 36

B.4.2.3. Supermolecular assemblies 36

B.4.2.3.1. Liposomes and ISCOMS 37

B.4.2.3.2. Ty:virus-like (Ty:VLP) particles 38

B.4.2.3.3. Self-aggregating viral proteins 38

B.4.2.3.4. Solid matrix-antibody-antigen (SMAA) Complexes 39

B.5. Genetic Immunisation 41

B.6. Mucosal Immunisation 42

B.6.1. Biodegradable polymer microspheres (microcapsules) 42

B.6.2. Live, recombinant bacterial vectors 43

B.6.3. Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT) 44

C. SIMIAN IMMUNODEFICIENCY VIRUS (SIV) 47

C.1. SIV, Protective Immunity and Vaccination 49

D. PROJECT OBJECTIVES 50

MATERIALS AND METHODS 51

1. RECOMBINANT DNA TECHNIQUES 51

1.1. Plasmids, Bacterial Strains and Culture Conditions 51

1.2. Synthesis of Synthetic Oligonucleotides 51

1.3. Construction of Plasmid pTRH-Pk 53

1.3.1. Preparation of plasmid DNA 53

1.3.2. Estimation of the concentration and purity of DNA in the plasmid preparation 55

1.3.3. Cleavage of plasmid DNA 55

1.3.4. Horizontal agarose gel electrophoresis 55

1.3.5. Purification of cleaved plasmid by electroelution 56

1.3.6. DNA ligation 57

1.4. Preparation and Transformation of Competent Bacteria 57

1.5. Preparation of L-Agar Plates 58

1.6. Screening for pTRH-Pk Recombinants 58

1.7. Storage of Colonies 59

2. ANTIBODIES 59

2.1. Purification of MAb SV5-P-k by Affinity Chromatography Techniques 60

3. SDS-PAGE AND WESTERN BLOTTING 60

3.1. Preparation of Polyacrylamide Gels 60

3.2. Preparation of Samples for SDS-PAGE and Electrophoresis 61

3.3. Coomassie blue staining 61

3.4. Western blotting and autoradiography 62

4. GM1-ENZYME LINKED IMMUNOSORBENT (GM1-ELISA) ASSAY 63

5. IMMUNE-PRECIPITATION ASSAYS..... 63

6. CONCENTRATION OF LTB-Pk..... 64

6.1. Concentration of LTB-Pk by Ammonium Sulphate Precipitation..... 64

6.2. Concentration of LTB-Pk by Ultrafiltration and in situ Dialysis..... 65

7. PURIFICATION OF LTB-Pk..... 68

7.1. Purification of LTB-Pk by Hydrophobic Interaction Chromatography using a Phenyl Superose Matrix on a Fast Protein Liquid Chromatography (FPLC) System..... 68

7.2. Purification of LTB-Pk by Hydrophobic Interaction Chromatography using a Bio-Rex Macroprep® t-Butyl Support..... 68

7.3. Purification of LTB-Pk by Ion-Exchange Chromatography using a Bio-Rad 70 Support..... 69

8. EXPRESSION AND PURIFICATION OF RECOMBINANT SIV PROTEINS..... 70

8.1. Plasmids..... 70

8.2. Expression of His-SIV-Pk Proteins in E.coli..... 70

8.3. Purification of Recombinant SIV Proteins by Ni^{2+}-NTA Affinity chromatography..... 71

8.4. Expression of GST-Pk..... 71

9. IMMUNOGENICITY STUDIES..... 72

9.1. Mice and Immunisations..... 72

9.2. Measurement of Immune Sera by the Enhanced Chemiluminscence (ECL) Assay..... 72

9.3. Measurement of the Secretory Response by ECL assay.....74

9.4. Lymphocyte Proliferation Assays..... 74

9.4.1. Preparation of splenocytes..... 74

9.4.2. Lymphocyte stimulation and measurement of DNA synthesis..... 75

9.5. Cytokine ELISA Assays 75

9.5.1. Preparation of culture supernatants 75

9.5.2. Detection of IL-2, IL-5 and IFN-γ 76

9.6. FACscan analysis 76

9.7. Enzyme-linked immunospot (ELISPOT) for the detection of antibody secreting cells 77

9.7.1. Preparation of lung cells 77

9.7.2. Preparation of spleen cells 78

9.7.3. Detection of antibody secreting cells (S.F.C.) 78

RESULTS 80

SECTION A. EXPRESSION AND CHARACTERISATION OF LTB-Pk 80

A.1. Introduction 80

A.2. Construction of Expression Vector pTRH-Pk 80

A.3. Comparison of the Expression and Cellular Localisation of LTB-Pk in E.coli and Vibrio sp.60 80

A.4. Oligomerisation of the Altered B Subunits 83

A.5. Binding of LTB-Pk to the Glycolipid, GM1-Ganglioside 84

A.6. Optimising the Expression of LTB-Pk 86

A.7. Stability of LTB-Pk Following Expression in Vibrio sp. 60 90

SECTION B. CONCENTRATION AND PURIFICATION OF LTB-Pk 92

B.1. Introduction 92

B.2. Concentration of LTB-Pk 92

B.2.1. Concentration and partial purification of LTB-Pk by ammonium sulphate precipitation 93

B.2.2. EDTA inactivation of protease activity; protection of the Pk-fusion 95

B.2.3. Concentration of LTB-Pk by ultrafiltration 98

B.3. Purification of LTB-Pk 99

B.3.1. Purification of LTB-Pk using a phenyl superose support

and FPLC..... 99

B.3.2. Purification of LTB-Pk by hydrophobic chromatography using a Macro-prep® t-butyl support..... 101

B.3.3. Purification of LTB-Pk by ion-exchange chromatography using a Bio-rex® cation-exchange support..... 104

B.4. Functional Properties of LTB-Pk Purified by Ion-Exchange Chromatography and the Stability of the Pk-epitope..... 105

SECTION C. EXPRESSION AND PURIFICATION OF His-SIV-Pk PROTEINS and CONSTRUCTION OF SIV-MAb-LTB COMPLEXES..... 108

C.1. Introduction..... 108

C.2. Construction and Expression of Recombinant His-SIV-Pk Proteins..... 109

C.3. Purification of His-SIV-Pk Proteins by Nickel Afinity Chromatography..... 109

C.4. Construction of SIV-MAb-LTB Complexes..... 111

(a) Construction of SIV-MAb complexes..... 111

(b) Purification of SIV-MAb complexes..... 111

(c) Linkage of LTB-Pk to purified SIV-MAb complexes..... 113

C.5. Binding of SIV-MAb-LTB complexes to GM1-ganglioside..... 115

SECTION D. SYSTEMIC IMMUNOGENICITY OF LTB-Pk..... 115

D.1. Introduction..... 115

D.2.Systemic Immunogenicity of LTB-Pk..... 116

SECTION E. SYSTEMIC IMMUNOGENICITY OF PARTIALLY PURIFIED p17-MAb-LTB COMPLEXES..... 121

E.1. Introduction..... 121

E.2. LTB-Pk Enhances the Immune Response to Recombinant SIV Antigens in vivo. 121

E.3. Characterisation of the Antibody Response to His-p17-Pk and LTB-Pk 127

E.4. p17-MAb-LTB Complexes Prime the Immune System to SIV 128

E.5. Induction of Cell-Mediated Immunity to His-p17-Pk by Immunisation with p17-MAb-LTB Complexes 130

E.6. FACscan and Cytokine Analysis of the Proliferating Cell Type 134

SECTION F. MUCOSAL IMMUNOGENICITY OF PARTIALLY PURIFIED p17-MAb-LTB COMPLEXES 138

F.1. Introduction 138

F.2. LTB-Pk Does Not Potentiate the Immune Response to His-p17-Pk Following Intranasal Immunisation 138

F.3. Instability of His-p17-Pk in Saliva 144

SECTION G. MUCOSAL IMMUNOGENICITY OF PARTIALLY PURIFIED p27-MAb-LTB COMPLEXES 146

G.1. Introduction 146

G.2. Resistance of His-p27-Pk to Salival Degradation 146

G.3. p27-MAb-LTB Complexes Can Induce Mucosal and Systemic Immunity to the Recombinant SIV Protein Following Intranasal Inoculation 149

G.4. Analysis of the Mucosal Response to His-p27-Pk and LTB-Pk in Intranasally Vaccinated Mice 153

(a) Measurement of secretory antibody 153

(b) ELISPOT analysis 153

G.5. Cell-mediated Immune Response 158

SECTION H. SYSTEMIC IMMUNOGENICITY OF HIGHLY PURIFIED p27-MAb-LTB COMPLEXES 163

H.1. Introduction 163

H.2. Purified p27-MAb-LTB Complexes Can Prime the Systemic Immune System to SIV 163

DISCUSSION 169

1 EXPRESSION OF LTB-Pk 169

2. CHARACTERISATION OF LTB-Pk 171

3. CONCENTRATION OF LTB-Pk 172

4. PURIFICATION OF LTB-Pk 174

5. SYSTEMIC IMMUNOGENICITY OF LTB-Pk 177

6. SYSTEMIC IMMUNOGENICITY OF SIV-MAb-LTB COMPLEXES 178

7. MUCOSAL IMMUNOGENICITY OF SIV-MAb-LTB COMPLEXES 182

8. AREAS OF FUTURE INVESTIGATION 186

REFERENCES 188

LIST OF FIGURES

FIGURE 1. Molecular interactions in antigen-specific T cell recognition of antigen-presenting cells 11

FIGURE 2. MHC class I presentation pathway 13

FIGURE 3. MHC class II presentation pathway 14

FIGURE 4. Solid matrix-antibody-antigen (SMAA) complexes 40

FIGURE 5. plasmid pTRH101R 52

FIGURE 6. Sequence of the positive and negative oligomucleotides 52

FIGURE 7. Concentration of LTB-Pk from *Vibrio sp.*60 cultures by ultrafiltration 66

FIGURE 8. DNA linker coding for the Pk tag 82

FIGURE 9. Expression and cellular localisation of LTB-Pk 82

FIGURE 10. Oligomerisation of LTB-Pk 85

FIGURE 11. Affinity of LTB-Pk for GM1-ganglioside 85

FIGURE 12. Effect of IPTG concentration and temperature on the expression of LTB-Pk in *Vibrio sp.*60 and *E.coli* 87

FIGURE 13. Influence of induction times on the expression of LTB-Pk 89

FIGURE 14. Stability of LTB-Pk following expression in *Vibrio sp.*60 91

FIGURE 15. Ammonium sulphate precipitation of LTB-Pk from *Vibrio sp.*60 cultures 94

FIGURE 16. Cleavage of the Pk-tag from LTB-Pk following ammonium sulphate precipitation 94

FIGURE 17. Stability of LTB-Pk following ammonium sulphate precipitation and dialysis against EDTA 97

FIGURE 18. Immune-precipitation of LTB-Pk 97

FIGURE 19. Protein purification prolfile by phenyl superose hydrophobic chromatography 100

FIGURE 20. Purification of LTB-Pk 103

FIGURE 21. Oligomerisation of LTB-Pk following purification by ion-exchange chromatography 106

FIGURE 22. Binding of purified LTB-Pk to GM1-ganglioside 107

FIGURE 23. Immune precipitation of purified LTB-Pk..... 106
FIGURE 24. Purification of His-SIV-Pk antigens..... 110
FIGURE 25. Construction of SIV-MAb-LTB complexes..... 112
FIGURE 26. Sub-saturation of MAb SV5-P-k..... 114
FIGURE 27. Binding of SIV-MAb-LTB complexes to GM1..... 114
FIGURE 28. Comparative analysis of the LTB-Pk-based vaccines..... 117
FIGURE 29. Immunogenicity of LTB-Pk..... 119
FIGURE 30. Comparative analysis of His-p17-Pk-based vaccine formulations..... 123
FIGURE 31. Induction of systemic immunity to His-p17-Pk..... 125
FIGURE 32. Induction of immunity to p17 from SIV-infected cell lysates..... 129
FIGURE 33 (a). Lymphocyte proliferative response to His-p17-Pk..... 132
FIGURE 33 (b). Lymphocyte proliferative response to LTB-Pk..... 133
FIGURE 34. FACscan analysis of His-p17-Pk stimulated cells..... 136
FIGURE 35. Serum antibody responses to His-p17-Pk and LTB-Pk following intranasal immunisation..... 140
FIGURE 36. Antibodies to His-p17-Pk and LTB-Pk in the saliva of intranasally vaccinated mice..... 142
FIGURE 37. Instability of His-p17-Pk in saliva..... 145
FIGURE 38. Stability of His-p27-Pk in saliva..... 148
FIGURE 39. Stability of p27-MAb-LTB complexes after exposure to salival proteases..... 148
FIGURE 40. Induction of serum immunity to His-p27-Pk and LTB-Pk by intranasal administration of p27-MAb-LTB complexes..... 150
FIGURE 41. Induction of systemic immunity to SIV following intranasal immunisation with p27-MAb-LTB complexes..... 152
FIGURE 42. Antibodies in the saliva of p27-MAb-LTB intranasally vaccinated mice specific for His-p27-Pk or LTB-Pk..... 154
FIGURE 43 (a). Lymphocyte proliferative response to His-p27-Pk..... 160
FIGURE 43 (b). Lymphocyte proliferative response to LTB-Pk..... 161
FIGURE 44. FACscan analysis of recombinant PHA, p27 and LTB-Pk stimulated immune cells..... 162

FIGURES 45 (a) & (b). Antibody response to His-p27-Pk after immunisation with purified p27-MAb-LTB complexes..... 165

FIGURE 46. Detection of antibodies to SIV p27 and p57..... 168

LIST OF TABLES.

TABLE 1. Concentration of LTB-Pk in relation to time..... 89

TABLE 2. Serum titres to LTB-Pk and GST-Pk..... 120

TABLE 3. Serum titres to His-p17-Pk in immune mice..... 126

TABLE 4. Cytokine production by stimulated recombinant p17-primed lymphocytes..... 137

TABLE 5. Serum antibody responses to His-p17-Pk following intranasal immunisation..... 140

TABLE 6. Antibodies to His-p17-Pk and LTB-Pk in the saliva of intranasally vaccinated mice..... 142

TABLE 7. Antibodies in the sera of mice intranasally immunised with p27-MAb-LTB complexes..... 152

TABLE 8. Antibodies in the saliva of p27-MAb-LTB intranasally vaccinated mice specific for His-p27-Pk or LTB-Pk..... 154

TABLE 9. Number of SFC secreting antibodies specific for His-p27-Pk..... 157

TABLE 10. Antibody titres to His-p27-Pk following intraperitoneal immunisation with purified recombinant p27-based vaccine formulations..... 167

INTRODUCTION.

Vaccination remains one of the most effective strategies for protecting man against viral infections. Vaccination specifically primes the cells of the adaptive immune system, the B and T cells, which enable them to respond quicker and with greater magnitude upon subsequent infection, and thereby, decreasing the severity of the disease.

Vaccination technology has advanced remarkably over the years, principally due to the developments in DNA technology which have now made it possible to construct subunit vaccines that contain only the epitopes important in the priming of B and T cells. This project, has adopted the subunit approach, with the aim of developing subunit vaccines that prime the immune system to the Simian Immunodeficiency Virus (SIV), and potentially Human Immunodeficiency Virus (HIV).

One of the fundamental aspects in the design of any vaccine, is to have a clear understanding of the adaptive immune responses, the role they play in the control of virus infections and the molecular events that lead to their activation.

The objective of the first two sections of this introduction is to present a generalised review of the adaptive immune system, its role in the control of viral infections, and how our understanding of this system is influencing vaccine design. In later sections of the introduction, a brief review of SIV is presented and some of the current vaccination strategies under investigation in the control of the virus infection are discussed. Finally, in the last section of the introduction, the major aims of this project are presented.

A. Adaptive Immune Responses To Acute Viral Infections.

A.1. Humoral Immunity to Viral Infections.

Immunoglobulins (Igs or antibodies) secreted from activated B cells are the effector molecules of humoral immunity. Immunoglobulins can exist in two microenvironments: as soluble products in the extracellular fluid (e.g. blood, tissue fluid or mucosal secretions) or expressed on the membranes of B cells where they function as antigen-specific receptors. In either environment, the immunoglobulin shares the same basic four chain structure and antigenic specificity. All immunoglobulin molecules are multifunctional, in that they can bind to specific antigenic epitopes through their variable N-terminus and interact with effector molecules through their more conserved Fc regions [Hilschman and Craig, 1965].

Antibodies are secreted from plasma cells, which are the principal effector cells of humoral immunity. Antibodies can control viral infections in different ways. For example, they can bind to specific components on the surface of the virus particle preventing infectivity (virus neutralisation), they activate the complement system which can lead to viral cytolysis and uptake by macrophages. Antibodies may target virus infected cells for lysis by either the complement pathway or through antibody-dependent-cellular cytotoxicity (ADCC). Thus, antibodies have a principal role in preventing viral infection of the cells.

The extent to which humoral immunity contributes to the overall protection against infection, is dependant on the actual infecting viruses. For example, both primary and secondary infections with vesicular stomatitis virus are controlled by neutralising antibodies [Gobet *et al.*, 1988] and the spread of enteric viruses from the portal of entry is more severe in B cell deficient patients [Rager-Zisman and Allison, 1973]. In contrast, humoral immunity seems to play only a minor role in the control of lymphocytic choriomeningitis virus (LCMV) [Zinkernagel *et al.*, 1976; Byrne *et al.*, 1984].

A.1.1. Classification of antibodies.

The basic unit of an immunoglobulin molecule is a four chain structure composed of two identical light (L) chains and two identical heavy (H) chains. The difference in structure of the amino and carboxy termini is reflected in the functions of the two regions. There is high sequence variability at the amino termini of both the heavy (V_H) and light (V_L) chains and this allows immunoglobulins to recognise and bind to a vast variety of structures. The conserved C-terminal (Fc region) interacts with effector cells (e.g. macrophages) and molecules (e.g. complement).

Nine classes of antibodies have been described in man (IgM, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgG_4, IgE, IgD, IgA_1 and IgA_2) eight classes in murine systems (the same classes as in man but there is only one IgA class), the classes being distinguishable from the different structures of their conserved C-terminal regions. In addition each class of antibody can exist in two forms, as free antibody secreted from plasma cells or as bound antibody that acts an antigen-specific receptor on the B cell surface. Both secreted and bound antibodies from a given cell are identical in their specificity for a particular antigen, only varying in their C-terminal region. Membrane bound immunoglobulins contain an additional transmembrane and cytoplasmic anchoring domain. (for a review of antibody structure and function, see Male *et al.*, 1987).

A.1.2. Activation of the B cell.

Naive B cells that have not previously been exposed to antigen, circulate through the body as small resting lymphocytes that are in the G_o phase of the cell cycle and bear membrane IgM and IgD molecules. Upon antigenic stimulation, resting B cells undergo a round of proliferation, termed clonal expansion creating many progeny B cells with antibodies having identical antigenic specificity. B cell progeny then differentiate into either memory cells or effector (plasma) cells. B cell proliferation and differentiation occur in organised lymphoid structures called lymph nodes, and is tightly regulated (reviewed by Clarke and Ledbetter, 1994). Antigenic stimulation is usually not enough on its own to induce B cells to proliferate, stimulation of the receptor facilitates the B cell to be more

receptive to signals secreted from activated T helper cells that are required for proliferation.

During B cell proliferation, progeny B cells undergo a process called class switching, whereby the class of antibody produced changes (for review of immunoglobulin class switching, see Snapper and Mond, 1993). Different classes of antibody have different effector roles. For example, the pentameric structure of IgM makes it very suitable to activate the complement system by the classical pathway, IgG can target viruses for lysis onto FcR bearing macrophages, IgA antibodies are principal effectors in the mucosal associated lymphoid tissue and IgE antibodies play a major role in allergic reactions. The class of antibody that is most effective in viral infections is unknown. However, studies by Coutelier *et al.*, [1987] have shown that infection of mice with various DNA and RNA viruses induces a striking dominance in the IgG_{2a} isotype. IgG_{2a} antibodies could be advantageous for clearing viruses as, because in contrast to IgG_1 and IgG_3, they can activate both the alternative and classical pathways of complement [Klaus *et al.*, 1979]. Macrophages also have distinct receptors for IgG_{2a} [Unkeless *et al.*, 1981] and IgG_{2a} antibodies are reported to be better mediators of ADCC than IgG_1 and IgG_{2b} antibodies [Steplewski *et al.*, 1985]. Whether such findings are relevant to man is unknown, as few studies have been conducted.

A.1.3. Generation of diversity of the B cell receptor.

One point that is paramount in considering the design of vaccines is to understand how B cells recognise antigens. The binding of foreign antigens to variable domains on antibodies present on the surface of the B cells represents the initial step in the sequence of events leading to activation of B cells and the secretion of effector immunoglobulins. The diversity in the number of antigenic structures capable of being recognised by immunoglobulin receptors on B cells, is a result of two processes unique to B cells- somatic recombination and hypermutation. Immunoglobulin polypeptide chains are encoded in multiple gene segments scattered along the chromosome of the germ-line genome [Hozumi and Tonegawa, 1976; Tonegawa *et al.*, 1977], the actual number of gene segments varying between species. For example, in murine systems, the H chain germ-line DNA contains a cluster of 100-200 variable (V_H) region segments [Givol *et al.*, 1981], a cluster of 12

diversity (D_H) segments [Schilling *et al.*, 1980] and a cluster of 4 functional joining (J_H) segments [Gough and Bernard, 1981]. In B cells, one each of the V_H segments, D_H segments and J_H segments are recombined to form a complete V_H region-coding DNA segment [Sakano *et al.*, 1979; Early *et al.*, 1980]. Because the V_H region is encoded by three gene segments and the joinings can occur in various combinations, the potential diversity generated by this rearrangement is enormous. A similar process occurs in the generation of the variable region of light chains, (for review on antibody somatic rearrangment see Tonegawa, 1983). This somatic recombination greatly diversifies the genetic information carried in the germ-line genome, and ultimately, the structure of the antigen-binding site on the immunoglobulin molecule. However, although such diversification in the structure of the antigen-binding site increases the number of antigens capable of being recognised, adversely, it tends to create receptors with low affinity [Fearon, 1993]. To overcome these problems, following primary stimulation and during clonal expansion of the B cells, mutations are introduced somatically into the immunoglobulin gene at an exceedingly high rate [Brack *et al.*, 1978; Bernard *et al.*, 1978]. B cells bearing high affinity receptors are then selected when antigen becomes limiting and differentiate into either memory B cells or antibody secreting plasma cells.

A.1.4. Determinants recognised by the B cell receptor.

There have been several attempts to distinguish the minimum epitope that can be recognised by immunoglobulins, in the hope of designing subunit vaccines. Many of the approaches have employed the use of short synthetic peptides that compete with native protein to bind specific antibody. Protein antigenic determinants that are recognised by immunoglobulin receptors have been classified as either sequential or conformational [Barlow *et al*, 1986]. Sequential determinants, sometimes referred to as linear or continuous epitopes, consist of amino acid residues that occur locally in the polypeptide chain. In contrast, conformational determinants are discontinuous, consisting of residues that occur far apart in the polypeptide chain, but are juxtaposed upon folding of the protein molecule. Most protein epitopes recognised by immunoglobulin receptors are thought to belong to this

latter category [Barlow *et al.*, 1986], since epitope accessibility is crucial for immunoglobulin interaction and most accessible epitopes are positioned at 'corners' of the folded polypeptide chain [Novotny *et al.*, 1986]. In support of this proposal, X-ray crystallographic images of a complex between lysozyme and a monoclonal antibody specific for the protein [Amit *et al.*, 1986] revealed that the epitope recognised by the specific monoclonal antibody was composed of two stretches of amino acid residues, namely residues 18-27 and 116-129, that came together upon folding of the protein molecule. In addition the size of the antigen binding site on immunoglobulin molecules is large, approximately 700 Å [Amit *et al.*, 1986] and few continuous epitopes of this size have ever been found [Barlow *et al.*,, 1986].

Several algorithms have been proposed to determine where B cell epitopes exist on proteins [Novotny *et al.*, 1987; Blundell *et al.*, 1987; van Regenmortel, 1989(a), 1989 (b)]. These include identification of regions that are buried within the protein i.e. hydrophobic regions and those regions which are hydrophilic and therefore are likely to be on the surface of the protein and thus, potentially antigenic. Although such programs are good at identifying some potentially important sites, they also fail to predict others and only result in a 50-55% efficiency in identifying the epitopes on the primary sequences [Pellequer *et al.*, 1991; van Regenmortel *et al.*, 1989(a)]. A classic example was demonstrated by Daniel and colleagues [1994] who showed that the combination of nine epitope prediction algorithms failed to identify critical epitopes necessary for protection against neurotropic murine coronavirus.

A.1.5. Evasion of the humoral response.

The specificity of the interaction between antibodies and their specific antigenic determinants can have serious implications in vaccine design. For example, vaccine formulations that denature the proteins of interest may not induce the appropriate immune response. Viruses themselves can thwart attempts to produce efficient vaccines by having many antigenic serotypes. Thus to produce a successful vaccine to many viruses, it may be

necessary to incorporate multiple epitopes into the vaccine to deal with virus antigenic variation.

A second problem in the design of vaccines, is that not all antibodies are actually protective and indeed, some may be harmful. It has been noted that for several viruses, e.g. Feline leukaemia viruses [Nick *et al.*, 1990], flaviviruses [Fagbami *et al.*, 1988] and dengue virus [Halstead *et al.*, 1988], that both neutralising and enhancing antibodies exist. For these viruses, subneutralising concentrations of antibodies can cause an enhancement of infection, via binding of antibody-virus complexes to cellular Fc receptors and thus facilitating the attachment of virus particles to target cells. Some antibodies against murine retroviruses have also been shown to enhance viral replication *in vitro* [Legrain *et al.*, 1986].

A.2. Cell-Mediated Immune Control of Viral Infections.

The replication of a virus is dependent on its ability to infect host cells and utilise the host's protein synthesising machinery. Thus, for part of its life cycle, a virus is located intracellularly and as a consequence, is hidden from humoral immune system. To overcome this problem, the immune system has evolved a second-line of defence that is concerned with recognising foreign intracellular antigens; cell-mediated immunity.

Cell-mediated immunity is characterised by T lymphocytes (T cells), which are quite distinct from their B lymphocyte counterparts in both their effector mechanisms and antigen recognition requirements. Rather than recognising free antigen, T cells have receptors (TcRs) that recognise and interact with peptide fragments of degraded proteins, presented on the surface of antigen presenting cells (APCs), in association with host cell molecules, termed major histocompatibility complex (MHC) proteins [Townsend *et al.*, 1989].

Different classes of T cells exist, and can be phenotypically distinguished by the type of cluster differentiation marker (CD) they carry on their cell surface [Knapp *et al.*, 1989]. Whereas $CD4^{-}CD8^{+}$ T cells interact with peptides presented in association with MHC class I molecules [Norment *et al.*, 1988], $CD4^{+}CD8^{-}$ T cells recognise peptides

bound to MHC class II molecules [Gay *et al*,. 1987] (see fig. 1). This classification of T cells into $CD8^+$ and $CD4^+$ cells, in part, reflects the different effector functions of the two T cell subsets. For example, $CD8^+$ T cells are normally referred to as having cytolytic activity (cytotoxic T lymphocytes; CTL), in that they actively destroy target cells. $CD4^+$ T cells, upon activation, secrete soluble substances called lymphokines [Stevens *et al.*, 1988; Balkwill *et al.*, 1989] that modulate the immune response, e.g. they provide the appropriate signals that allow precursor cytototoxic T cells to differentiate into effector cells [Jennings *et al.*, 1991; Horvat *et al.*, 1991], and provide help for B cells to proliferate and differentiate into antibody secreting plasma cells [Stevens *et al.*, 1988; Snapper and Paul, 1987]. However, these functions are not mutually exclusive. In this respect, certain $CD8^+$ T cells secrete lymphokines [Fong and Mossman, 1990]. Similarly, some subtypes of $CD4^+$ T cells exhibit cytolytic activity [Del Prete *et al.*, 1991(a); (b)].

T cells have a critical role to play in the resolution of viral infections and the ability to design vaccines that actively prime T cells to a particular virus have the potential to be very successful. This means that detailed information on the intracellular routes of antigen processing and the relative importance of $CD8^+$ as opposed to $CD4^+$ T cells in the resolution of viral infections is important in the design of vaccines. In the following sections, the cells, molecules and molecular mechanisms leading to the activation of T cells is presented.

A.2.1. Antigen Presenting Cells (APCs).

One of the major requirements in T cell activation, is that viral proteins must be processed and degraded into short peptide fragments that can associate with MHC class I or II molecules. Such events are performed in antigen presenting cells (APCs). Virtually all nucleated cells bear MHC class I molecules and therefore have the potential, following viral infection, to act as APC for $CD8^+$ T cells. In contrast to the ubiquitous expression of class I MHC molecules, MHC class II molecules have a more restricted distribution, being principally expressed on the surface of specialised cells of the haemapoietic lineage [Cresswell *et al.*, 1987] i.e. B cells, macrophage and dendritic cells. Considering the central

role $CD4^+$ T cells play in the immune system, this restricted distribution of MHC class II molecules may serve as a regulatory mechanism in the immune system.

There have been several attempts to establish whether one cell-type predominates as an APC for activation of naive T cells, in the hope of designing vaccines that can be targeted directly at the appropriate APC. Although such research has greatly facilitated understanding of the minimum requirements for a cell to function as an APC, which include the ability of the cell to internalise antigen [Stockinger *et al.*, 1992; Gasselin *et al.*, 1992], the processing capabilities of the cell [Vidard *et al.*, 1992], the level of expression of adhesion molecules [Sanders *et al.*, 1988], the surface charge of the cell [Boog *et al.*, 1989] and the provision of additional co-stimulatory signals efficient for T cell activation [Mueller *et al.*, 1989; Lui and Janeway, 1992], there is no conclusive evidence that one cell type is more efficient than another for activating naive T cells. For example, although studies on B-cell deficient mice, which contain fully functional macrophage and dendritic cells, suggest that antigen-specific B cells are the predominant activators of unprimed T cells [Kurt-Jones *et al.*, 1988], Macatonia and colleagues [1989] suggest that dendritic cells are the most efficient APC. Indeed, the general opinion is that dendritic cells are the most efficient APC for activating unprimed T cells [reviewed by Knight and Stagg , 1993].

A.2.2. The Major Histocompatibility Complex (MHC).

The major histocompatibility complex molecules have a prominant role to play in T cell activation, since peptides must be presented to T cells in association with these molecules. Sequencing studies have shown that both MHC class I and II molecules are highly polymorphic [Coligan *et al.*, 1981; Brown *et al.*, 1988], there being numerous haplotypes. In 1987 (a), Bjorkman and colleagues, resolved the X-ray crystallographic image of an MHC class I molecule, HLA-A2 and, such studies revealed that class I MHC molecules have a deep groove running between the $\alpha 1$ and $\alpha 2$ domains, whose sides are composed of α-helical structures, and the floor consists of eight anti-parallel β-pleated sheets. Most of the polymorphism seen in class I MHC molecules is located within the groove [Bjorkman *et al.*, 1987(b)] and it was proposed that this groove contained the

peptide binding site. Since these initial studies, the X-ray images of several other MHC class I and class II molecules have become available [Garret *et al.*, 1989; Brown *et al.*, 1993] and have supported the observation that peptides are located in the grooves of MHC molecules. There have been several attempts at sequencing the amino acids lining the peptide binding grooves of various MHC molecules and such studies have shown that the binding grooves contain six subsites or 'pockets'. Comparative studies have demonstrated that within haplotypically similar MHC molecules these subsites are present in similar locations and contain almost identical amino acids. Yet, in MHC molecules of different haplotypes, the size, shape and location of the pockets are dissimilar. These observations have led to the suggestion that the peptide sequences that bind to certain MHC molecules are restricted, in that, only peptides that are capable of interacting with the amino acids located at particular regions in the groove of the MHC, will be selected for presentation to T cells. Such an observation would imply that similar peptide motifs would bind to haplotyically similar MHC molecules, a suggestion that has won support from the work of Falk and colleagues, 1991. In a series of experiments, naturally occurring peptides were eluted from murine MHC class I H-2K^d and H-2K^b, then sequenced. It was found that peptides eluted from H-2K^d molecules had similar peptide sequences that were distinct from those eluted from H-2K^b molecules. It is now generally accepted that peptide-binding motifs exist and thus it may eventually be possible to engineer peptide vaccines that bind to particular MHC haplotypes.

A.2.3. The T cell receptor (TcR).

T cells recognise peptide-MHC complexes through their surface T cell receptor [TcR (see Fig.1)]. Most peripheral T cells bear TcR composed of α β polypeptide chains and such receptors are extremely polymorphic. The polymorphism of the T cell receptor occurs from somatic rearrangement of gene segments in an analogous fashion to that which occurs with antibodies [Chothia *et al.*, 1988; Claverie *et al.*, 1989, reviewed by Davis, 1990].

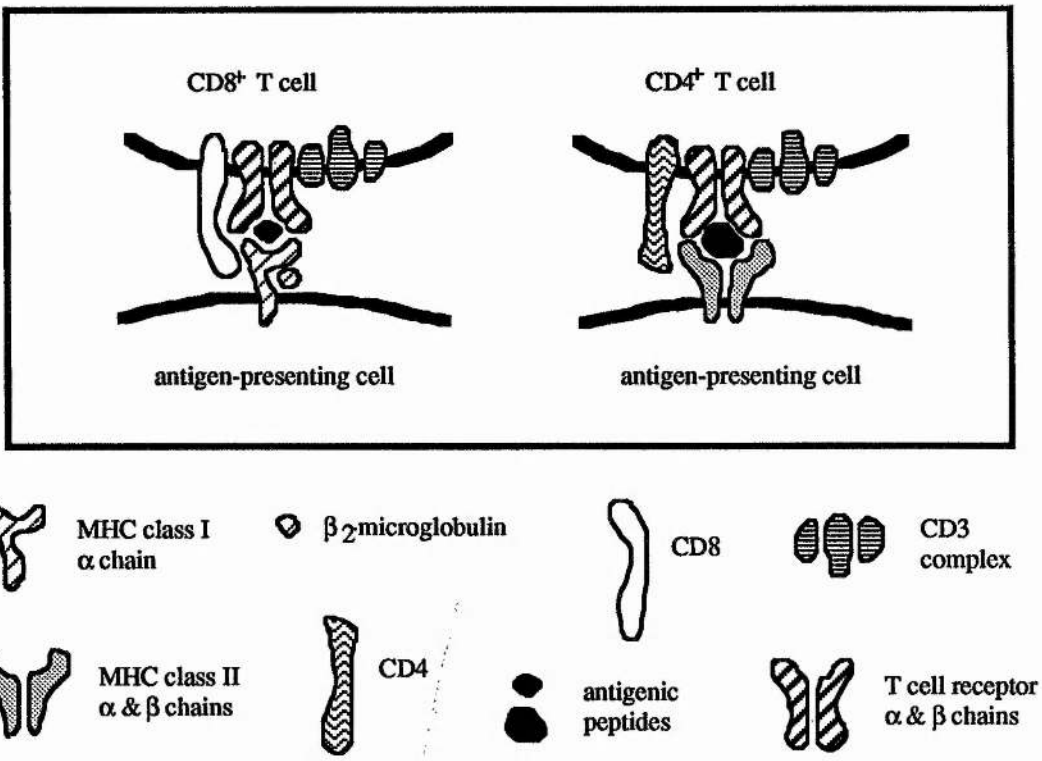


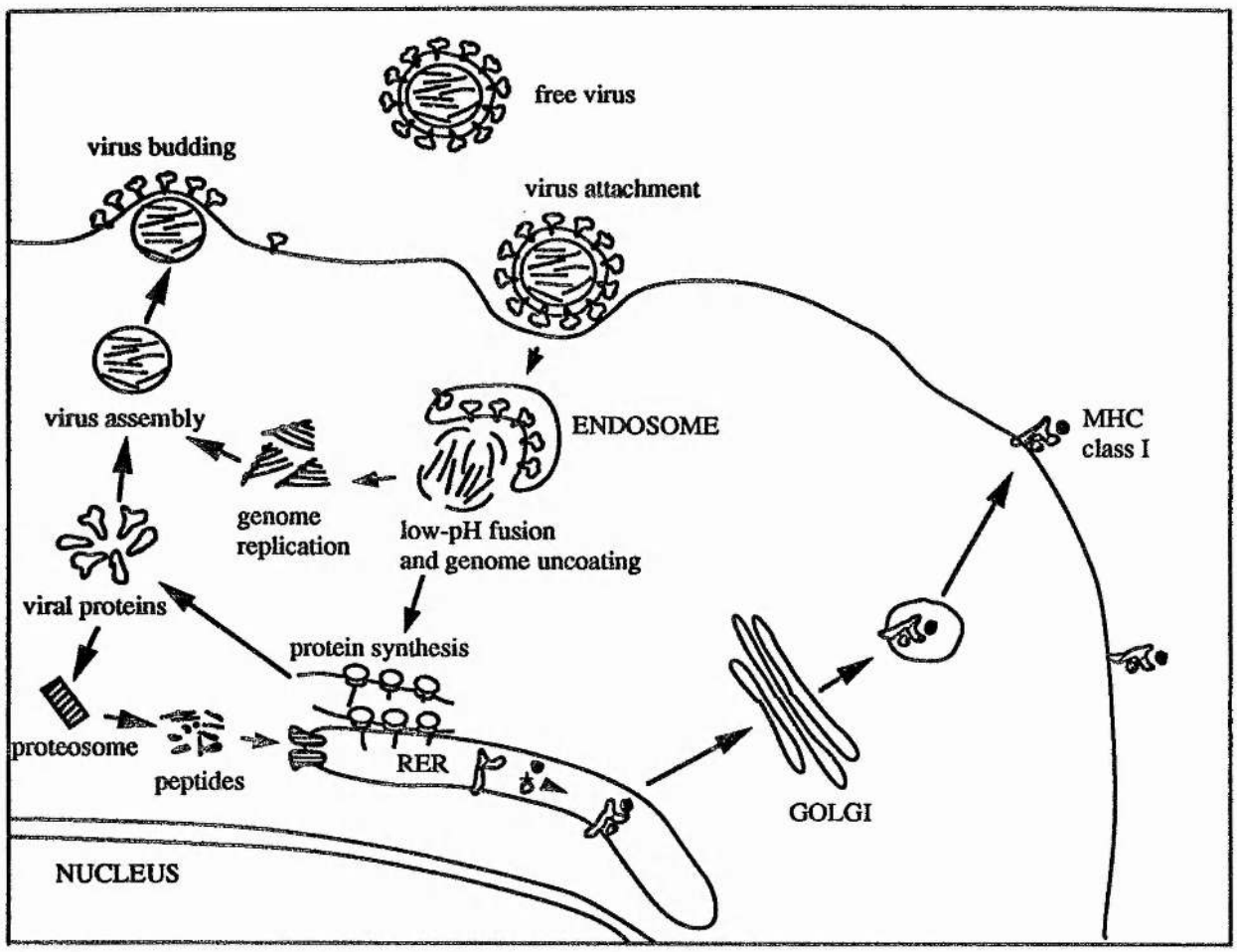
Figure 1. Molecular interactions in antigen-specific T cell recognition of antigen-presenting cells.
The left-hand side of the figure depicts the interactions during the recognition of peptides associated with MHC class I molecules by TCR on a $CD8^+$ T cell (usually a cytotoxic T lymphocyte). The right-hand side of the figure illustrates the recognition of MHC class II-peptide complexes by a $CD4^+$ T cell (a helper or possibly a cytotoxic T cell). The key determinant of T cell specificity is the T cell receptor (TCR). The TCR is closely associated with the CD3 complex and either a CD8 or CD4 molecule. The CD3 complex is involved in signal transduction across the plasma membrane following TCR engagement and so are most likely the CD8/CD4 molecules. (Taken from Hanke and Randall, 1994).

Despite the fact that αβ TcR bearing T cells are extremely sensitive to low antigen concentrations (only 30-100 peptide-MHC complexes on APC are required for T cell triggering [Demotz *et al.*, 1990; Harding *et al.*, 1990], the affinity of TcRs for such complexes are very low [Matsui *et al.*, 1991; Weber *et al.*, 1992]. Such observations suggest that TcR-ligand interactions are not the driving force for conjugate formation between T cells and target cells [Singer *et al.*, 1992] and it has been proposed that multiple adhesion molecules expressed on both the APC and the T cell, many of which show extremely high affinities for their ligands, must mediate the event [Springer *et al.*, 1990]. For example, it is known that the binding of CD4 to MHC class II [Doyle, *et al.*, 1987] and CD8 to MHC class I [Norment *et al.*, 1988] allows the recognition process to progress more efficiently and with less antigen [Gabert *et al.*, 1987].

A.2.4. Pathways of Processing and Presentation of Antigens.

Proteins have to be processed into peptide fragments that are capable of interacting with both the T cell receptor and the appropriate MHC molecule. Two pathways for processing proteins have been suggested depending on whether the resulting peptide associates with MHC class I or class II molecules; the endogenous and exogenous pathways respectively [Figs. 2 & 3 repectively, Germain, 1986]. In the endogenous pathway, newly synthesised proteins are degraded in the cytosol and then transported to the endoplasmic reticulum (ER) where they complex with newly synthesised MHC class I molecules (Fig.2). The role that the ER plays in this endogenous pathway is highlighted by the ability to arrest MHC class I presentation by use of pharmaceutical products like Brefeldin A, that specifically prevent egress of molecules from this cellular compartment [Nuchtern *et al.*, 1989]. In contrast to the endogenous pathway, the exogenous pathway is concerned with the degradation of proteins into peptide fragments that subsequently associate with MHC class II molecules. Such presentation can be inhibited by lysomotropic agents (e.g. chloroquinine), but not Brefeldin A, that specifically act by increasing the pH of endosomes, suggesting that these cellular compartments play a critical role in the degradation of the proteins (Fig.3). The exact compartment where complexing between peptides and MHC

Class I presentation pathway

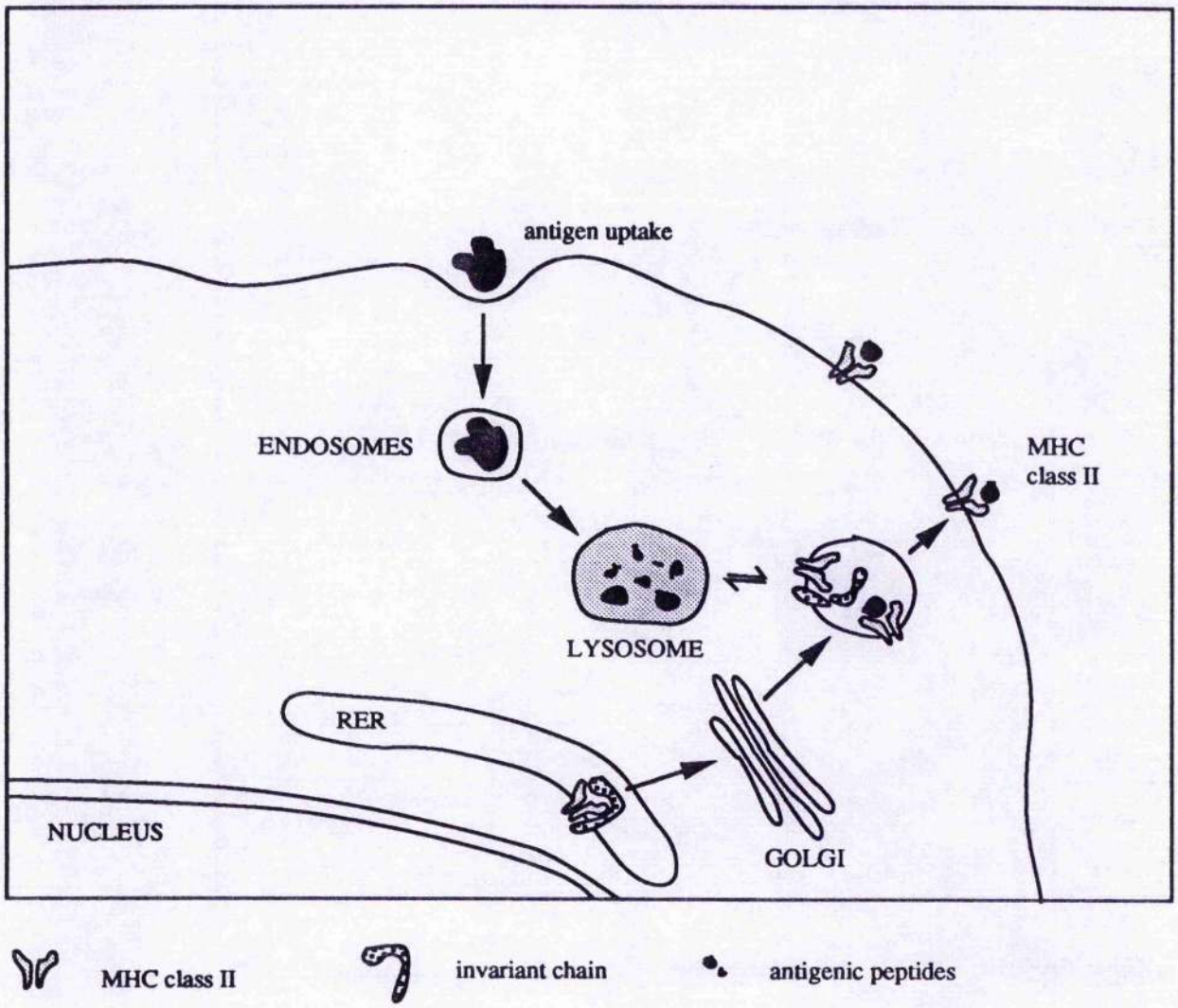


RER rough endoplasmic reticulum

(Modified from Hanke and Randall, 1994)

Figure 2. MHC class I presentation pathway.
The diagram schematically illustrates the life cycle of a virus (in this case influenza virus) and how the virus proteins can enter the endogenous (MHC class I) antigen processing pathway in an antigen-presenting cell. Following partial proteolytic degradation, certain virus-derived peptides associate with MHC class I molecules. The MHC-peptide complexes are then transported to the cell surface where they are specifically recognised by T cells bearing T cell receptors.

Class II presentation pathway



RER rough endoplasmic reticulum

(Modified from Hanke and Randall, 1994)

Figure. 3. MHC class II presentation pathway.
The diagram schematically illustrates the life cycle of a virus (in this case influenza virus) and how the virus proteins can enter the exogenous (MHC class II) antigen processing pathway in an antigen-presenting cell. Following partial proteolytic degradation, certain virus-derived peptides associate with MHC class II molecules. The MHC-peptide complexes are then transported to the cell surface where they are specifically recognised by T cells bearing T cell receptors.

class II complexes occurs is unknown, but it has been noted that the endosomes cross the pathway leading to the expression of MHC class II molecules on the surface of the cell. Thus it has been suggested that complexing with MHC class II molecules occur in endosomes themselves [for a review on antigen processing and presentation, see Hanke and Randall, 1994].

Recent evidence suggests that the processing of antigens to class II or class I presentation may not be so straightforward. For example, exogenous (non-replicating) antigens that gain direct entry into the cytosol can be presented in association with MHC class I molecules [Moore *et al.*, 1988]. It has also been suggested that there may be specialized APC *in vivo* capable of capturing exogenous antigen and processing it for MHC class I presentation. It has also been demonstrated that certain endogenously synthesised proteins can be presented in conjunction with MHC class II molecules [Jarquemada *et al.*, 1990; Michalek *et al.*, 1992]. Although this latter route of presentation has been attributed to the secretion of endogenously synthesised antigen, followed by its subsequent re-capture by antigen-specific receptors and thus internalisation into the endosomal/lysosomal pathway [Yurin *et al.*, 1989; Bikoff *et al.*, 1989; Jin *et al.*, 1988; Polydefkis *et al.*, 1990; Eager *et al.*, 1989]], there is evidence to suggest that there are two unique pathways in the processing of endogenous antigen to MHC class I- and II-restricted T cells. For example, Jarquemada *et al.* [1990] have demonstrated that infection of a human B cell-line expressing MHC class I HLA-A2 molecules and MHC class II MHC HLA-DR1 molecules with recombinant vaccinia virus containing influenza A virus matrix protein (M1), resulted in both MHC class II and class I-restricted T cell lysis of the cell-line. Such MHC class II-restricted lysis could be inhibited by chloroquinine, whereas class I-restricted lysis could not. The converse was true when Brefeldin A was added to the assay, suggesting that endogenous antigens can gain access to endosomal/lysosomal compartments via an intracellular route, leading to the processing and presentation in a class II-restricted manner.

A.2.5. Immune regulation; influence of the immune response genes.

Soon after the discovery of MHC restriction it became apparent that many viruses and cellular antigens were recognised in association with some but not other MHC molecules [Blanden *et al.*, 1975; Simpson and Gordon, 1977; Mullbacker and Blanden, 1978]. Furthermore, some peptide-MHC complexes were immunogenic in one strain of mice but not another. For example, for three different viruses, (vaccinia virus, sendai virus and cross-reactive influenza A virus) mice of the K^bD^b haplotype respond to virus determinants in association with H-$2D^b$, whereas mice of the K^kD^b haplotype did not respond to virus antigens in association with D^b [Zinkernagel, 1978; Doherty *et al.*, 1978] . However, using K^kD^b lymphocytes *in vitro*, it was shown that D^b was capable of presenting the CTL epitope to D^b-restricted CTL, thus excluding the notion that the non-responders fail to present antigen in association with D^b. Data presented by Hill *et al* [1993] is also compatible with the concept of 'immunodominance' where the responding T-cell population appeared to focus the immune response on a limited number of MHC-peptide complexes. Thus, in the presence of certain more strongly immunogenic antigens, responses to other apparently more weakly immunogenic antigens was not seen.

The means by which some antigens are favoured over others is unclear, but could include (a) affinity of T cell clones for antigen; (b) T-cell precursor frequency and (c) the concentration of the relevant epitope on antigen presenting cells. In any case, the phenomenon applies that there is competition between T cell clones, either for binding antigen presenting cells, for helper factors or other nutrients. Thus, response to weaker antigens is only observed if the dominant antigen is limited. The existence of immune response hierarchies, however, is of more than passing academic interest. Recombinant DNA technology now makes it possible to manufacture vaccines expressing limited antigenic determinants of the virus of interest. The mere identification of a determinant as immunogenic in the presence of a particular MHC molecule (e.g. in *vitro*) does not guarantee a response *in vivo* whenever that particular MHC-peptide combination is presented. Potential vaccine vectors, such as recombinant vaccinia virus carry their own

strong antigenic determinants for all class of immune response. Whether an engineered determinant is able to induce an immune response may be dependent on its place in the immune response hierarchy, generated by the vector carrying it and the MHC genes of the responding animals.

A.2.6. Viral escape of cell-mediated responses.

The importance of cytotoxic $CD8^+$ T cells in the control of viral infections can be highlighted by the strategies some viruses use to prevent recognition by $CD8^+$ T cells. For, example, certain viruses can interfer with MHC class I surface expression. One of the best documented examples of inhibition of MHC class I presentation by viral infection is that of adenovirus E19 protein on MHC class I transport [Andersson *et al.*, 1985]. E19 interacts with MHC class I molecules within the ER and prevents transport of these molecules to the cell surface, thereby preventing presentation of the viral T-cell epitopes on the cell surface of the infected cells. Similarly, human cytomegalovirus (HCMV) exerts a strong inhibitory effect on the cell surface expression of class I MHC [Browne *et al.*, 1990]. However, although downregulation of class I would seem an obvious mechanism to evade CTL, this has been shown to improve recognition of viral infected cells by natural killer (NK) cells [Karre *et al.*, 1986; Harel-Bellan *et al.*, 1986]. Other ways in which viruses may avoid recognition by class I-restricted CTLs is to infect cells (e.g. neuronal cells) which do not express class I MHC; a mechanism that is thought to correlate with persistent infection by LMCV. Some viruses may also be capable of subverting CTL memory by the selection of CTL escape mutants. For example, a longitudinal study of HIV seropositive patients revealed there were fluctuations in the specificity of the cytotoxic T cells. This was matched by variability in proviral gag DNA epitope sequences and some of the viral variants were not recognised by CTL [reviewed by Levy, 1993].

A.2.7. Cellular immune responses to viruses and implications for vaccine design.

As indicated above, cell-mediated immune responses to viruses are complex. For example, some CTL responses are class I restricted, whereas others are class II restricted. The importance of these two classes in the resolution of virus infection can be different for different viruses. For example, following influenza infection, the majority of CTLs are class I restricted [Bourgault *et al.*, 1989], yet after measles infections class II-restricted CTLs appear to predominate [Jacobson *et al.*, 1984; 1989]. This difference may, in part, be related to the mechanism of viral entry [Long and Jacobson, 1989]. As a consequence, vaccine technology may have to be engineered to provoke the appropriate class of CTL.

A.3. Deleterious versus Protective responses.

It is evident from the preceding sections that B and T cells have important roles in the control of viral infections. However, knowledge of the relative importance of B and T cells in the resolution of a viral infection is critical in the design of vaccines to that virus. Although for some viruses it may be possible to induce immunity to some viral diseases by inducing high levels of neutralising antibody to a restricted number of antigenic determinants, (as is the case for foot and mouth disease; Francis *et al.*, 1988), for many diseases it may be necessary to induce both antibody and cell-mediated immunity. It is clear from studies on immunodeficient patients that the relative importance and balance of humoral and cell-mediated immune responses may vary from one virus infection to another. For example, measles infection normally proves lethal in children with defects in cell-mediated immunity, yet, children with agammaglobulinaemia can contract and survive measles in a relatively normal fashion [Burnet, 1968; Ewan and Lachmann, 1982]. While is some cases it is well established that CTLs play an important role in the protection against infection, for some viruses inappropriate CTLs may contribute to the viral pathology. For example, transfer of CTLs specific for respiratory syncytial virus (RSV) into immunodeficient mice can increase the pathology following subsequent infection [Cannon *et al.*, 1988].

In other cases, B cell responses may be harmful. For example, enhancement of viral infectivity due to antibodies directed at particularly determinants has been reported in several cases, including human immunodeficiency virus [Bolognesi, 1989]. It has also been reported that antibody-induced antigenic modulation may be an important factor in the establishment of persistent infections *in vivo* [for review of persistent infection see Randall and Russell, 1990]. A fine balance may thus exist between the induction of protective immunity and the induction of immunity that will lead to detrimental responses upon subsequent infection with virulent virus. Consequently, vaccines may have to be designed that induce not only the appropriate type of immunity, i.e. humoral versus cell-mediated, but immunity is directed at epitopes that are protective and not epitopes that are deleterious.

A.4. Long lived immunity.

One of the primary goals of vaccination is to create long term memory cells capable of responding to their specific antigen, upon subsequent encounters, swifter and with greater magnitude. However, little is known about the generation of immunological memory. Several concepts have been proposed to account for immunological memory including long-lived memory cells [Strober and Dilley, 1973], different thresholds of stimulation of naive and lymphocyte memory cells (which may not be long lived) due to altered surface expression of adhesion molecules [Sanders *et al.*, 1988] periodic stimulation with cross-reactive antigens [Beverley, 1990], re-stimulation by recurrent infections [Mims, 1987] or antigen persistence in specialised reservoirs [Tew *et al.*, 1980; Tew and Mandel, 1979] causing continual stimulation of memory clones. These latter concepts have been proposed because recent evidence suggesting that non-replicating pathogens need to persist if long term memory is to be maintained for both B cells [Gray and Skarvall, 1988] and T cells [Gray and Matzinger, 1991]. This leads to the question of how a non-replicating antigen persists. At present the only known repositories for antigen are on the surface of follicular dendritic cells (FDCs) found in the B lymphocyte follicles of lymph-nodes, which can maintain native antigen as immune-complexes for months or years [Tew *et al..*, 1990] and specifically present antigen to B cells. If FDCs are subsequently found to be the only

cell-type that can store antigen for long periods of time, then an inescapable conclusion of such a finding is that the survival of T lymphocyte memory must be dependant on the production of antibody and on the presence of memory B cells [Gray *et al.*, 1991]. However, for $CD8^+$ T cells, evidence suggests that the clonal burst is more important than antigen persistence in the maintenance of $CD8^+$ memory [reviewed by Doherty *et al.*, 1994]

A.5. Mucosal Immunity to Viruses.

The mucosal surfaces in the body, represented by the respiratory, gastrointestinal and urinogenital tracts, are readily infected by a wide variety of RNA and DNA viruses. However, parental immunisation schedules that evoke efficient systemic immunity, rarely induce protection at the mucosal surfaces. Such evidence has led to the proposal that the mucosal immune system is distinct from the systemic immune system and highlighted the need for the development of vaccines that induce protective immunity at mucosal surfaces. However, the development of effective mucosal vaccines has been hindered because, for many years, understanding the immune mechanisms operative at mucosal surfaces has proved an enigma. In part, this difficulty in analysing the mucosal system has been due to the inherent difficulties in the isolation and characterisation of lymphoid cells in the various mucosal-associated tissues. However, since the major route for the entry of Human Immunodeficiency Virus (HIV) is through the urino-genital tract and rectum, vaccines that are likely to induce protective immunity to HIV will have to prime both the mucosal and systemic systems. This has re-focused attention once more on deciphering the intricacies of the mucosal immune system. In this section, a brief review on the current understanding of the immune cells and effector mechanisms operative at the mucosal surfaces is presented.

A.5.1. Evidence of a common mucosal immune system.

Interest in designing vaccines that stimulate mucosal immunity stems from observations that immunisation of one mucosal site often leads to detectable immune responses at distant mucosal sites [Mestecky *et al.*, 1987] (and potentially systemic

immunity too). This seeding of distal mucosal sites is likely to be the result of trafficking of locally stimulated mucosal B cells to distinct sites, where they reside as IgA plasma cells actively producing antibodies. Such trafficking of antiviral IgA producing cells can occur in the gastrointestinal tract mucosa to the respiratory mucosa and perhaps vice versa [Waldman *et al.*, 1986; Chen *et al.*, 1987; Hirabayashi *et al.*, 1990]. Although this common mucosal system exists, it appears to be relatively inefficient at protecting sites not directly stimulated with antigen [Nedrud *et al.*, 1987].

A.5.2. Antigen uptake at the mucosal surfaces.

Accumulating data over the last few years, has indicated that the mucosal immune system is anatomically and functionally divided into separated regions, one where antigens are encountered, processed and initial B- and T-cell triggering occurs (called inductive sites) and areas where immune cells actually function (called effector sites) [McGhee *et al.*, 1992]. In man, the major inductive sites are putated to be the Peyer's patches of the gastrointestinal tract [McGhee *et al.*, 1992; Mestecky and McGhee, 1987] and the tonsils in the upper respiratory tract [Bernstein *et al.*, 1994], with the lamina propria and salivary/mammary glands constituting the effector sites in the gastrointestinal and repiratory tracts, respectively. Most studies on the uptake of antigens at the mucosal surfaces have therefore been concerned with events occurring at these putative mucosal inductive sites. Such regions are equipped with specialised antigen uptake cells, termed follicular-associated epithelium (FAE) or M cells [Owens and Jones, 1974]. M cells efficiently pinocytose/endocytose soluble and insoluble antigens [Bockman and Cooper, 1973; Pappo and Ermak, 1989], as well as micro-organisms [Owen *et al.*, 1986] and sometimes serve as specific sites of viral entry e.g. reovirus types 1 and 3 [Wolf *et al.*, 1981] infect mucosal surfaces by binding to specific receptors on the M cell surface. Upon uptake, antigens are rapidly transported, intact, in a 'central hollow' or 'pocket region' to the underlying antigen presenting cells which subsequently process the antigen for presentation to T cells [Owen *et al.*, 1977; 1986]. Antigens that are capable of adhering to M cells are more efficiently transported than their non-adherent counterparts [Neutra *et al.*, 1987]. Such evidence has led to investigations into

novel antigenic delivery systems that promote antigen uptake by M cells. Several of these novel strategies are discussed in later sections.

The epithelial cells of the effector regions can also act as portals of antigen entry [reviewed by McGhee and Kiyono, 1993]. In particular, soluble vaccines are particularly adept at reaching the underlying lymphocytes following endocytosis by the overlying epithelium. Alternatively, vaccines may transverse tight junctions between epithelial cells and intact antigen could trigger B- and T-cell responses. However, the actual role epithelial cells play in the induction of mucosal immunity is something that remains to be ascertained.

A.5.3. Humoral immunity at the mucosal surfaces.

The protection of the mucosal surfaces from viral infection is generally assumed to be conferred by virus-specific secretory antibodies of the IgA isotype. A preliminary site for IgA-committed B cells seems to be the Peyer's patches [Craig and Cebra, 1971]. Following activation, the B-cells migrate from Peyer's patch through the thoracic duct and back to the effector sites of the lamina propria where they differentiate into IgA secreting plasma cells (reviewed by McGhee and Kiyono, 1994].

The majority of IgA produced enters mucosal secretions via epithelial transcytosis mediated by the polymeric Ig receptor (also known as the transmembrane secretory component) [Solari *et al.*, 1985]. The mucosal response to primary viral infection is rapid and can occur within three days of infection [Rubin *et al.*, 1983]. However, this response is not sustained [Bishop *et al.*, 1990; Coulson *et al.*, 1990], which may in part explain why mucosal surfaces, as opposed to peripheral system, are more readily re-infected with mucosal viruses.

The predominant role that IgA antibodies are thought to have in response to viral infection is to neutralise the virus, though it has been noted that unlike IgG antibodies, IgA are poor activators of complement and ADCC [Pfaffenbach *et al.*, 1982]. Although the exact mechanisms of how IgA neutralisation of viruses occurs is unknown, it has been proposed that IgA antibodies may decrease infectivity by aggregating viruses [Outlaw and Dimmock, 1990] or by blocking attachment of viruses to cell receptors [Outlaw and Dimmock, 1991]

or preventing penetration of attached viruses into the cells [Nguyen *et al.*, 1986]. Receptors for the Fc region of IgA have also been found on epithelial cells and thus, IgA may potentiate the clearance of viruses by boosting their uptake by epithelial cells and transport to the underlying lymphoid cells [Weltzin *et al.*, 1989]. IgA may also neutralise virus inside infected epithelial cells by inhibiting virus replication [Outlaw and Dimmock, 1990]. For example, experiments on Sendai virus have shown that virus in infected cells could be reduced by the addition of virus-specific IgA, presumably through the uptake of IgA into the cell by the polymeric receptor [Mazanec *et al.*, 1992].

In vivo responses support *in vitro* virus neutralisation studies. Mice passively immunised intranasally with IgA antibodies were protected from intranasal challenge with sendai virus [Mazanac *et al.*, 1992]. In a separate study of influenza virus, IgA polymeric monoclonal antibody to haemagglutinin administered systemically, was able to restrict virus replication in the upper respiratory tract of the mouse [Renegar and Small, 1991]

Despite the prominent role that the IgA response plays in the protection of the mucosal surfaces against viral infection, IgG antibodies have also been suggested as contributing to the overall reduction of infectivity at this site. In IgA-deficient patients mucosal production and secretion of IgG antiviral antibodies can compensate for the deficiency in the production and secretion of IgA [Ogra *et al.*, 1974]. Further, serum IgG antibodies, produced in response to respiratory virus infection, have been shown to restrict virus replication in the lung more effectively than in the trachea or nose [Ramphal *et al.*, 1979; Prince *et al.*, 1985]. Passively transferred IgG can also restrict replication of poliovirus to a greater extent in the throat than in the lower intestine [Bodian and Nathanson, 1960]. Much of the IgG response detectable at mucosal surfaces originates from the transudation of serum IgG into the mucosal site [Wagner *et al.*, 1987; Murphy *et al.*, 1982], though virus-specific IgG antibodies produced by the mucosa also contribute to the total antiviral activity in mucosal secretions [Ogra *et al.*, 1974; McBride and Ward, 1987]. The mechanism of antiviral activity of IgG antibodies in the mucosa is related to direct neutralisation as opposed to complement-mediated lysis or ADCC, a finding supported by the observations that RSV replication is restricted in the lungs of passively immunised

rodents depleted of complement and that $F(ab)_2$ fragments of IgG, can restrict pulmonary virus replication as effectively as whole IgG molecules [Reviewed by Murphy, 1994].

A.5.4. Epitopes recognised by B cells at mucosal surfaces.

Several studies have shown that the epitopes recognised by IgA antibodies and IgG antibodies are similar. For example, polyclonal IgA antibodies , like polyclonal IgG antibodies recognised the haemagglutinin of influenza virus [Clements *et al.*, 1986]; the gp70 (fusion) and gp90 (attachment) glycoproteins of respiratory syncytial virus [Murphy *et al.*, 1986]. However, the antigens of viruses may be modified by mucosal enzymes to expose new epitopes on the surface proteins of such viruses. Thus the immunogenicity of viruses that replicate at mucosal surfaces may differ from a parentally administered inactivated vaccine. For example, poliovirus VP3 protein is cleaved by intestinal enzymes following oral administration of live virus, thereby exposing unique epitopes on the protein that are recognised by IgA antibodies [Zhaori *et al.*, 1989].

A.5.6. Cell-mediated immunity at the mucosal surfaces.

Although most studies on protective immunity at the mucosal surfaces has centred on the role of the secretory antibody response, MHC class I-restricted $CD8^+$ cytotoxic T cells can both function as antiviral effector cells against viruses that infect the mucosal surfaces. For example, passive transfer of $CD8^+$ cytotoxic T cell clones to animals results in the restriction of influenza virus replication in mucosal epithelial cells [McDermott *et al.*, 1987]. MHC class II-restricted $CD4^+$ T cells have also been shown to have direct anti-viral activity [McDermott *et al.*, 1987; Taylor *et al.*, 1990] and are proposed to play a role in the generation of the secretory IgA antibody response, e.g. IL-5 and IL-6 secreted from activated $CD4^+$ Tcells are reported to be important in the switching of B cells to secrete IgA [Beagley *et al.*, 1988].

A.5.7. Intraepithelial lymphocytes.

As well as in the Peyer's patches and lymphoid structures of the gut, T-lymphocytes are found all along the murine intestinal mucosae. The most striking of these cells are the ones located between the epithelia- the intraepithelial lymphocytes (IEL). In the murine system, it has been suggested that T cells carrying the γδT cell receptor predominate here [Koning *et al.*, 1987; Kuziel *et al.*, 1987] (though recent evidence would dispute this [reviewed by Guy-grand, 1993]) and as such may be important in the immune surveillance of the epithelia. Indeed, the exclusive expression of the γδTcR by intraepithelial T lymphocytes and the fact that peripheral T-lymphocytes very rarely express this receptor, suggests that the T-lymphocytes in the γδTcR-expressing T lymphocytes specifically home to epithelial surfaces in the mucosal system and thus, they must have some critical role here [Janeway *et al.*, 1988].

Studies by Bonneville and colleagues, on the murine intestine, have demonstrated that such cells also carry the CD8+ molecule [Bonneville *et al.*, 1988] and are thought to be cytotoxic in nature [Ernst *et al.*, 1985; Klein *et al.*, 1986]. Also the presence of granules containing serine esterases in these intraepithelial cells is compatible with their proposed cytotoxic role [Guy-Grand *et al.*, 1991]. It has also been noted that intraepithelial cells are capable of secreting certain lymphokines, e.g. interleukin-2 (IL-2), -3 (IL-3), gamma interferon (IFN-γ), tumour necrosis factor-α (TNF-α) and TNF-β [Viney and McDonald, 1992; Barret *et al.*, 1992; Taguchi *et al.*, 1991].

However, the importance of these unique cells in the murine system does not seem to correlate with present findings in humans where the majority of gut intraepithelial T lymphocytes, like their peripheral T lymphocyte counterparts, bear the αβ T cell receptor, although they are also preferentially $CD8^+$ [Brandtzaeg *et al.*, 1989]. As yet unproved, two important functions have been ascribed to the gut intraepithelium T cells, namely MHC-restricted or non-restricted cytotoxicity [Ernst *et al.*, 1985] and suppression of mucosal hypersensitivity [Brandtzaeg *et al.*, 1988; Doherty *et al.*, 1992]. Nevertheless, the actual role these cells play in the surveillance of the mucosal surfaces and the ligands that they

recognise are still unknown. The importance of immunisation to prime intraepithelial cells is something that remains to be investigated.

B. Vaccination.

Multiple factors can influence the efficacy of vaccines namely: the types, number and association of antigens used, their shape and conformation, the timing of their administration, the addition or absence of adjuvants, and the immune status of the vaccinee. It is also important to stress that the route and dose of immunising antigen is important in the induction of T and B lymphocyte responses. Small amounts of antigen can fail to induce a response but too large a quantity can lead to immune tolerance to the antigen. In light of these principals, some of the advantages and disadvantages of various immunisation procedure that have been used to make vaccines, or are being proposed as methods of making novel vaccines, will now be briefly considered.

B.1. Live, attenuated viral vaccines.

Live, attenuated mutants of wild -type viruses, i.e. viruses that are capable of infecting host cells but do not induce the disease characteristic of native virus, offer one of the most attractive strategies for producing protective immunity against viral infections. Since attenuated viruses are capable of replicating *in vivo*, any viral protein has the potential to be presented to the immune system, thus, evoking a wide spectrum of humoral and cell-mediated immunity to both structural and non-structural viral proteins. In addition, attenuated viruses presumably infect the same types of cells as the wild-type virus, and thus could potentially give rise to T cell responses with individuals selecting the appropriate T cell epitopes depending on their MHC status. Also, some live attenuated viruses may be capable of persisting and restimulating the immune system at regular intervals which may obviate the need for booster inoculations.

However, despite the potential advantages of live, attenuated viruses as vaccines, there are a number of concerns about the safety of such vaccines. For example, the attenuation

process is normally achieved by culturing the native virus through a non-natural host [Sabin and Bougler, 1973]. This results in the virus accumulating many mutations, the mutant viruses that are shown to have the least virulence, in either experimental animals or in humans, being selected as potential vaccines. However, such a process makes it difficult to decipher what the mutations are and there is a danger that an attenuated virus may revert to its virulent form through subsequent mutations during the replication of the vaccine virus in the host cells [Almond *et al.*, 1985]. In addition, for certain live attenuated viruses, the persistence of the virus can be detrimental to the host. For example, the persistence of rubella virus in lymphocytes after vaccination has been linked with the onset of arthritis [Chantler, 1981]. Nevertheless, despite these problems, the efficacy of such live attenuated viruses as vaccines has been highlighted in a dramatic way by the global eradication of smallpox and control of yellow fever as well as the success of such vaccines in inducing protection against poliomyelitis, measles and rubella [Hilleman, 1985].

B.2. Whole, killed virus vaccines.

Live viruses can be inactivated by heat treatment and exposure to protein modifying agents. These so-called, killed virus vaccines, represent the second most traditional approach to immunisation. Killed viruses are incapable of replicating *in vivo*, thus presenting an initial advantage over their attenuated counterparts in that there is no danger of disease due to viral reversion. In addition, killed virus vaccines tend to be more stable, and therefore easier to store than attenuated vaccines. Despite these advantages, there are several disadvantages in the use of killed viruses as vaccines. For example, it is paramount that all infectious virus particles in the vaccine preparation must be inactivated- a point that is highlighted by incidents of paralytic disease in vaccinees given inactivated polio vaccine which was found to contain trace amounts of live polio virus [Nathanson, 1963; Peterson *et al.*, 1955]. There have also been reports of vaccinees given formalin-inactivated respiratory synctia virus (RSV) vaccine exhibiting serious lower respiratory tract complications upon subsequent natural infection with native RSV [Kapikian, 1969]. In

this case, and others [Rauh and Schmidt, 1965; Kim *et al.*, 1969; Fulginiti *et al.*, 1967; Orvell and Norrby, 1977], the process of inactivating the virus seems to be responsible for the adverse reactions. For example, studies in cotton rats revealed that formaldehyde treatment of RSV led to the cross-linkage and subsequent destruction of virus neutralising epitopes on the fusion and attachment glycoproteins of the virus [Prince *et al.*, 1986]. Subsequently, upon infection with native virulent virus, an unusually large antibody response was directed at non-protective epitopes on the viral glycoproteins, resulting in immune-complex deposition in the lungs and increased lower respiratory tract pathology. Thus great care must be taken during the inactivation process to ensure epitopes that are critical for protection against infection, are not damaged. Even if critical epitopes are preserved, it is possible that the viral proteins in killed virus vaccines will be preferentially presented to MHC class II-restricted T cells due to the lack of endogenous synthesis of the viral proteins. In this instance, it is unlikely that killed virus vaccine will be effective against viruses that are normally cleared by MHC class I-restricted cytotoxic T cells.

In addition to the problems mentioned above, the inability of killed virus vaccines to replicate, also means that larger doses of virus may have to be administered at regular intervals for the induction of long term protective immunity. Unfortunately, vaccines that have to be given in multiple inoculations are not only unattractive to the general public, but also increase the risk of hypersensitivity reactions.

B.3. Subunit vaccines; a reductive approach to vaccination.

Subunit vaccines, which are characterised by synthetic peptides and purified viral proteins, are an attractive and relatively new approach to vaccination. Obvious advantages in the use of subunit vaccines, are that they are non-infectious and that they contain only the proteins or epitopes important for inducing protective immunity. However, these advantages are offset by the disadvantage that such vaccines are generally non-immunogenic when administered on their own. This paradox has led to the search for suitable adjuvant formulations (e.g. alum, muramyl-dipeptide, Quil. A) or carrier vehicles (e.g. ISCOMS, microspheres, SMAA complexes; below) that can potentiate the

immunogenicity of the subunit vaccines by maximising presentation of the target viral antigens to the immune system. In the following sections, a general review on the more recent advances in vaccine technology is presented, but for the moment, the use of peptides and purified proteins, themselves, as immunogens is discussed.

B.3.1. Peptides.

Immunisation with short, synthetic peptides corresponding to the appropriate protective epitopes of a virus is an attractive vaccination strategy, since potentially, vaccines could be designed that stimulated whichever effector function was particularly beneficial in the protection against virus infection. Naturally, the first step in this approach necessitates the availability of an efficient detection system for identifying critical antigenic epitopes present on the native virus particle. Although rapid advances have been made in this area of immunology, such research has highlighted the problems in using synthetic peptides as human vaccines. For example, since most B cell epitopes tend to be discontinuous i.e. composites of amino acids from different regions within the same protein [Barlow *et al.*,, 1986], then peptides will have to be constructed that adopt an identical conformation to that of native viral proteins if they are to be effective [Satterthwaite *et al.*, 1989]. There have been attempts to overcome this problem by adding secondary structures to B cell epitopes. For example, the placement of cysteine residues at each end of a peptide sequence has been shown to facilitate the formation of disulphide bonds [Schulze-Gahmen *et al.*, 1985; Leonard *et al.*, 1990] and increased the immunogenicity of the B cell epitopes. Another novel strategy that has been shown to enhance the immunogenicity of B cell epitopes is the development of Multiple Antigen Peptide Systems (MAPS) [Tam, 1988]. This approach uses a small peptidyl core matrix bearing radially branching synthetic peptides as dendritic arms, to which the desired B cell epitopes can be linked.

However, other problems exist in the design of peptide vaccines for the stimulation of B cell responses; the necessity for T cell help for efficient B cell activation and antibody secretion, means that T cell epitopes may have to be coupled to the B cell epitopes, unless of course, the B cell epitopes themselves, contains sequences stimulatory

for T cells. In addition, the ability of certain viruses to antigenically vary important B cell epitopes, suggests that efficient vaccines will have to include multiple B cell epitopes. The development of peptide vaccines that stimulate T cells has its own set of problems. For example, the consequence of peptide selection by polymorphically distinct MHC in any population, means that peptide sequences that are immunogenic in one individual may not be in another. Consequently, peptide vaccines may need to contain multiple T cell epitopes [Rothbard, 1987].

B.3.2. Purified, recombinant viral proteins.

There are a number of potential advantages in using purified viral proteins as vaccines. For example, their non-infectious nature means they are unlikely to cause disease, and since the proteins contain multiple antigenic determinants, it is possible both B cell and T cell responses can be induced. Two practical requirements for purified proteins to be incorporated into vaccines are that they can be produced on an industrial scale (i.e. there is a need for suitable expression systems that can produce large quantities of recombinant protein that resembles natural protein) and that the purification scheme conserves sensitive conformational viral epitopes. The importance of a rigorous purification scheme is of particular importance for viral proteins expressed in continuous cell-lines, where the possibility of contaminating oncogenic DNA in the vaccine preparation, may lead to tumours upon vaccination. Prokaryotes, yeasts, baculoviruses and mammalian cells have all received attention as potential expression systems for viral proteins [Luckow *et al.*, 1988, Hanke *et al.*, 1994; Randall *et al.*, 1993], where the expression of viral proteins can be controlled by inserting viral genes downstream from strong constitutive or inducible promoters [Kleid *et al.*, 1981]. Yeasts have been particularly successful for the production of recombinant Hepatitis B virus surface antigens that have subsequently been used in wide-spread vaccination programmes.

Novel protein purification strategies are also currently being investigated. For example, through recombinant DNA technology it is possible to delete viral domains that normally anchor viral proteins in the cell membrane of the expressing cell, thereby

facilitating secretion of the protein and therefore making purification simpler [Lasky *et al.*, 1986; Whang *et al.*, 1987]. An alternative strategy, described by Hanke and colleagues (below), is to attach small, affinity tags to viral proteins which subsequently can be used to purify the recombinant viral protein on anti-tag affinity columns.

However, apart from a few general exceptions [Valenzula *et al.*, 1982; McAleer *et al.*, 1984], as with peptide-based vaccines, purified proteins on their own, tend to be poorly immunogenic *in vivo*, often having to be administered in conjunction with powerful adjuvants or coupled to carrier systems.

B.4. Novel strategies for potentiating the immune response to subunit vaccines.

The rapid advancement in the production of peptide and purified proteins as potential subunit vaccines, has been paralleled by developments in novel strategies to increase their immunogenicity. Current interest in this field of immunology, has centred on the development of improved adjuvant formulations or the manipulation of recombinant viruses and bacteria to express and present foreign viral proteins to the immune system. Naturally, such research has yielded a vast array of novel vehicles for potentiating the immunogenicity of subunit vaccines, and although each has its own particular merits, it is outwith the scope of this introduction to be able to describe them all in detail. Thus, the rest of this section is concerned with describing adjuvants and carrier systems, currently receiving the most attention.

B.4.1. Adjuvants.

Adjuvants, classically defined, are any substance that can augment the immune response to a particular antigen when it is administered at the same time as the antigen. There is a vast array of chemical, bacterial and immunological products that have been ascribed adjuvant properties [Penny et al., 1993; Bernstein *et al.*, 1993; Good *et al.*, 1988] and have recently been reviewed by Gupta and colleagues [1993]. However, most adjuvants are highly toxic, inducing several unpleasant side-effects, that make their use in

man unlikely. As such, there has been a large amount of research into new adjuvants that retain the adjuvanticity of existing formulations, but exhibit greatly restricted adverse reactions. A few of the more recent advances in human adjuvant research that show particular promise, are described below.

(a) Mineral compounds.

The use of alum (aluminium hydroxide and aluminium phosphate) as an adjuvant was described more than 60 years ago [Glenny, 1926] and even today, it is the only adjuvant licensed for use in man. Alum-based products can be prepared either by the precipitation or adsorption of the desired protein onto the mineral compound [Randall *et al.*, 1994], the latter strategy being more efficient at producing a uniform vaccine formulation. Although alum is generally considered a safe adjuvant, there are variable reports on its efficacy. For example, alum-adsorbed inactivated poliomyelitis virus was highly immunogenic *in vivo* [Butler, 1962] but adsorption of influenza A virus haemaglutinin onto alum had no enhancing effect in clinical trials [Davenport *et al.*, 1968]. Since the mode of action of alum is thought to be due to a depot formation [Leeling *et al.*, 1979] which delays resorption of the protein from the site of injection (thereby prolonging the period of antigenic stimulation), it is generally assumed that the efficacy of alum as an adjuvant for a particular protein will be dependant on how well that protein can adsorb to alum [Skea *et al.*, 1993 (a); (b)]. This may help explain the discrepancy in the clinical trials described previously (i.e. influenza haemagglutinin binds poorly to alum). There have also been reports that alum-based vaccines are weak inducers of cell-mediated immunity [Bomford, 1980], and as such, they may be inefficient against viruses whose replication is normally controlled by this arm of the immune system. There are other potential problems in the use of alum as an adjuvant. For example, it has been shown that following administration, alum can induce granulomatous formation at the site of injection [Walls *et al.*, 1977], as well as enhancing IgE production [Cogne *et al.*, 1986], and therefore it may have a possible role in increasing allergic disease [Bohler-Sommeregger *et al.*, 1986]. Also, the recent evidence that suggests aluminium compounds may be neurotoxic, leading to

neurological disorders like Alzheimer's disease [McLachlan *et al.*, 1989], has caused concern over the utility of alum in vaccine formulations.

Two alternatives to alum have recently been suggested; calcium phosphate [Goto *et al.*, 1993] and an octadecyl ester of tyrosine, octadecyl tyrosine hydrochloride (stearyl tyrosine) [Penny *et al.*, 1993]. Both have been shown to be stable, efficiently entrap proteins, can stimulate high levels of IgG (but little IgE), do not induce granulomatous formation and since they are composites of natural constituents of the body, are readily adsorbed [Relyveld et al., 1986; Penny *et al.*, 1993; Nixon-George *et al.*, 1990].

(b) MDP analogues.

Probably the most famous adjuvant is Freund's complete adjuvant (FCA). This oil-in-water emulsion contains mycobacteria in the oil phase, and has been shown to be a potent inducer of both humoral and cell-mediated immunity, including cytotoxic T cell responses in experimental animals. However, FCA is highly toxic inducing fever, cramps and necrosis at the site of injection, which makes it unsuitable for use in man. The active component of FCA has been linked to the presence of the mycobacteria, in particular the muramyl dipeptide (*N*-acetylmuramyl-L-analanyl-D-isoglutamine, (MDP)) component of the bacterial cell wall [Chedid *et al.*, 1976]. MDP itself is highly immunogenic but, like FCA, it is extremely toxic. This has led to several studies to determine if the immunopathological effects of MDP can be separated from the adjuvant properties. Such research has shown that a synthetic analogue of MDP, threonyl-MDP (t-MDP) exhibits adjuvant activity in experimental animals without inducing severe side-effects [Allison and Byars, 1986]. The potential of such adjuvants in man is currently under investigation.

(c) Cytokines.

Cytokines are also being exploited as potential adjuvants, in particular recombinant interleukin-2 (rIL-2) [Good *et al.*, 1988]. For example, injection of rIL-2 with the hepatitis B vaccine, induced humoral immunity to the viral surface protein in individuals who were normally non-responders to hepatitis vaccination [Meuer *et al.*, 1989; Kawamura *et al.*, 1985]. In contrast to these findings, immunisation of guinea-pigs with IL-2 and herpes

simplex virus-2 (HSV-2) induced an efficient cell-mediated response to HSV-2 glycoprotein D but had little effect on antibody production [Weinberg and Merigan, 1988].

However, despite the initial success of cytokines as adjuvants, it does not yet establish them as alternatives to conventional adjuvants like alum, and it is important to point out that cytokines can induce serious side-effects, even when administered in minor amounts. It may be, that in time, after further research into their effectiveness and toxicities, cytokines may have a specialist application in vaccine design, e.g. in overcoming hyporesponsiveness, or selectively inducing cell-mediated immunity.

B.4.2. Novel carrier systems.

In recent years there has been a major development in the construction of various polymeric presentation systems, that optimise the delivery of peptides and purified proteins to the immune system. There are two types of, so-called, carrier systems:- expression based and non-expression based. Live, recombinant viral and bacterial vectors are examples of the former category, whereas non-expression based carriers are characterised by immunostimulatory complexes (ISCOMS), liposomes, solid matrix-antibody-antigen (SMAA) complexes and Ty-particles to name but a few. Carriers may serve several functions, they may allow presentation of multiple epitopes of the target antigen and/or provide the necessary T cell epitopes for the provision of B cell help. One concern with carriers is the effect of carrier induced suppression, where pre-existing immunity to the carrier can downregulate the immunogenicity of the target antigen [Schutze *et al.*, 1985]. Thus, several areas of research into carrier systems for peptides or purified proteins, have relied on the development of novel synthetic carriers where no pre-existing immunity to the carrier exists. In the following sections, a general review of some of the current carrier systems that are under investigation as potential human vaccines are presented.

B.4.2.1. Live, recombinant viral vectors.

Several different viruses have been proposed as potential live carrier systems for viral proteins including vaccinia virus [Andrew *et al.*, 1991], avipox virus [Radaelli *et al.*, 1994; Radaelli and De Giuli Morghen, 1994], herpesvirus [Roizman and Jenkins, 1985], adenovirus [Johnson *et al.*, 1988], rotavirus [Redmond *et al.*, 1991], and poliovirus [Burke *et al.*, 1988; Evans *et al.*, 1989; Dedieu *et al.*, 1992]. Some viral vectors e.g. attenuated vaccinia and adenovirus replicate *in vivo*, whereas others e.g. poliovirus minireplicons and avipox, are engineered to have a restricted or abortive replication, but they all retain the ability to infect. Thus, upon infection, foreign proteins are expressed during the normal replication of the virus, thereby potentially inducing both humoral and cell-mediated immunity. However, certain problems exist in the use of live, recombinant viruses as carriers for viral proteins. For example, the size of the genome can restrict the amount of foreign genetic information that can be inserted into the vector. Viruses like, vaccinia, that contain a large 231 kilo base-pair genome, can accommodate high amounts of foreign DNA [Smith and Moss, 1983] either by inserting the desired sequence downstream of a defined vaccinia promoter sequence [Mackett *et al.*, 1982] or by insertion into a nonessential loci [Panicali and Paoletti, 1982; Perkus *et al.*, 1985; 1986]. In contrast, poliovirus, with its characteristic 7.7 kilo-base RNA genome, can only accommodate relatively small inserts [Burke *et al.*, 1988].

Live replicating viruses present the additional problem of finding suitably attenuated strains that can act as carriers. As examples, the gene product E1A of adenovirus can transform cells in culture, causing concern as to whether it may be oncogenic in man, and attenuated vaccinia virus strains currently available may induce neurological complications e.g. encephalopathy in immunosuppressed individuals, as well as leading to the exacerbation of dermatological conditions. Although new strategies for attenuating vaccinia virus have recently been described, e.g. animal studies have shown the insertion of foreign DNA into the thymidine kinase locus is accompanied by the attenuation of the recombinant virus, this does not necessarily mean that the virus will be attenuated in man [Buller *et al.*, 1985; Rodriguez *et al.*, 1989; Hu *et al.*, 1986]. Insertion

of cytokine genes into the genome of vaccinia virus, in particular IL-2, has also been reported as a novel strategy for attenuating the virus. This latter strategy having the additional benefit in that the expression of IL-2 in conjunction with the foreign viral protein, can over-ride non-responsiveness.

B.4.2.2. Live, recombinant bacterial vectors.

Mycobacterium bovis bacillus Calmette-Guerin (BCG), the human tuberculosis vaccine and attenuated strains of Salmonella have both been used as carriers for foreign proteins, the latter being used as a carrier for mucosal immunisation (see later). BCG has features that make it a particularly attractive live recombinant, bacterial vaccine vehicle; BCG, as with other mycobacteria, are excellent adjuvants, with a long-lived safety record in man. In addition, BCG engenders a long-lived immune response with a single dose, is heat stable, inexpensive to produce and one of the few vaccines that can be given at birth. Recent developments in genetic engineering have shown that it is possible to insert foreign genes into BCG [Jacobs *et al.*, 1987; Snapper *et al.*, 1988] and following vaccination, such expression of the resulting foreign proteins can induce both humoral and cell-mediated responses [Aldovini and Young, 1991], including MHC class I-restricted $CD8^+$ CTL. Strover and colleagues, [1991] showed that foreign genes inserted under the control of the heat shock promoter, could lead to high level expression of the foreign proteins, and as such only low doses of BCG were needed for immunisation purposes.

However, one problem with the use of BCG or any bacteria for that matter, is the inability to express glycosylated viral proteins in native conformation. Since viral glycosylated proteins normally contain the antibody neutralising domains, it is questionable whether any bacteria expressing the genes for such viral proteins will be capable of inducing immunity to these protective epitopes.

B.4.2.3. Super-molecular assemblies.

Several new approaches in the design of carrier systems for peptides and purified proteins have been concerned with the development of large molecular assemblies that

contain multiple copies of one or more foreign epitopes. The repeating units of the assembled carrier protein stimulate T lymphocytes efficiently and the use of an efficacious adjuvant can further increase both cell-mediated and humoral responses. A brief review of some of the most promising carrier systems is presented below.

B.4.2.3.1. Liposomes and ISCOMS.

Liposomes and immunostimulatory complexes (ISCOMS) are examples of lipid-based carrier systems. Liposomes are membrane vesicles, formed by the dispersion of phospholipids in aqueous media that possess several interesting properties required for *in vivo* carriers. For example, they are relatively non-toxic, exhibit low immunogenicity and are biodegradable [Snyder and Vannier, 1984]. ISCOMS on the other hand, are cage-like particles of 30-40nm in size that are composed of the saponin Quil A, cholesterol and amphipathic antigen in a molar ratio of 1:1:1 [reviewed by Morein, 1988]. Both liposomes and ISCOMS can stimulate a wide spectrum of immune responses. For example, liposomes have been reported to protect against influenza [Friede *et al.*, 1994] and can induce MHC class I-restricted $CD8^+$ CTL responses [Reddy *et al.*, 1992]. In part this adjuvanticity of liposomes is related to the number of layers [Shek *et al.*, 1983], their charge [Allison and Gregoriadis, 1974], and their composition [Heath *et al.*, 1976].

Likewise, ISCOMS have been shown to generate immunity to a wide variety of viral proteins, including influenza [Lovgren, 1988], measles [DeVries *et al.*, 1988], rabies [Osterhaus *et al.*, 1986], glycoprotein 340 from Epstein Barr Virus [Morgan *et al.*, 1988] and the glycoprotein 120 from HIV [Pyle *et al.*, 1989]. In slight contrast to the safety of liposomes, ISCOMS are still restricted to veterinary use. A problem with ISCOMS, that prevent their use in man, is the presence of the toxic adjuvant Quil A. Nevertheless, several attempts are being made to design non-toxic versions of Quil A that retain the adjuvant properties of the parent molecule.

B.4.2.3.2. Ty: Virus-Like Particles (Ty:VLP).

Certain yeast proteins that spontaneously associate into virus-like particles (VLP) [Mellor *et al.*, 1985] are also under consideration as carriers of viral proteins [Griffiths *et al.*, 1991]. Such an approach is typified by the Ty:VLP system wherein a foreign DNA sequence is fused to the TYA gene and can then be expressed in yeasts [Adams *et al.*, 1991]. For example, fusion of the principal antibody neutralising domain, V3, of Human Immunodeficiency Virus-1 (HIV-1) to Ty:VLP resulted in an effective MHC class I-restricted $CD8^+$ CTL response to the viral antigen [Layton *et al.*, 1993]. Ty:VLP particles are also showing promise as mucosal vaccines [Lehner *et al.*, 1992].

B.4.2.3.3. Self-aggregating viral proteins.

The hepatitis B virus core antigen (HBcAg) and surface antigen (HBsAg) are examples of self aggregating viral proteins that have been suggested to have potential carrier activity for foreign antigens [Francis *et al.*, 1990]. For example, HBcAg spontaneously self aggregates into characteristic 27nm particles [Almeida *et al.*, 1971], and chimeric core particles can be created by the insertion of peptide sequences onto the N- and C-termini of HBcAg [Clarke *et al.*, 1987; Stahl *et al.*, 1989]. The HBcAg particle itself is highly immunogenic [Hoofnagle *et al.*, 1973], presumably due to the virus proteins' polymeric nature as well as the presence of a number of well-defined helper T cell epitopes [Milich *et al.*, 1987] and this may contribute to the adjuvant properties of HBcAg for fused foreign antigens.

An example of HBcAg as a carrier system is presented by the study of Francis and colleagues [1990]. Fusion of the VP2 proteins from human rhinovirus type 2 to HBcAg, resulted in both humoral and cell-mediated immunity to VP2 following parental administration. Low levels of serum antibody was also detected following intranasal or oral vaccination, so such HBcAg-fusions may have potential as mucosal vaccines. Similarly, fusion of the V3 domain from HIV-1 to either the N- or C-terminal of HBcAg resulted in the induction of neutralising antibodies to the HIV [Von Brunn *et al.*, 1993]. MHC class I-restricted $CD8^+$ CTL can also be induced by immunisation with the target

epitopes fused to hepatitis virus antigens, e.g. HBsAg [Schlienger *et al.*, 1992], suggesting that these novel self aggregating viral proteins have the potential to induce all arms of the immune response.

B.4.2.3.4. Solid matrix-antibody antigen (SMAA) complexes.

A novel strategy to vaccine design has been the development of solid matrix-antibody-antigen complexes [reviewed by Randall, 1989]. The basis of this scheme is to use monoclonal antibodies, linked to a solid matrix (e.g. heat-killed*Staphylococcus aureus* , an organism that can bind to the Fc region of IgG_{2a} antibodies via the Protein A determinant of the bacteria wall, thereby leaving the antigen binding arms of the antibody free to complex with its specific antigen, or the adjuvant alum), to first purify the antigen and then to use the SMAA complexes as immunogens (see Figure 4). SMAA complexes themselves are particulate and can induce both vigorous antibody responses as well as class I-restricted CTL [Randall and Young, 1988; Randall *et al.*, 1988]. The potential advantages and disadvantages of such a method of antigen presentation have been discussed elsewhere [Randall, 1989]. Principally the major advantage of such a system is that the complexes are extremely easy to produce and it is relatively simple to incorporate multiple virus antigens, including structural and non-structural proteins into the SMAA complex. Also antigens can be derived from virtually any source, be it expression vectors or virus infected tissue culture cells, and can simply be incorporated into the SMAA complexes. As alternative to conventional adjuvants it has also been suggested that it may be possible to induce an immune response to antigens by targeting the binding of antigens directly to particular host cells [Randall, 1989]. This could be achieved by coupling the antigens to monoclonal antibodies that recognise cell surface determinants, such as immunoglobulins [Kawamura and Berzofsky, 1986] or MHC class I and class II antigens [Snider and Segal, 1987; 1989; Carayanniotis and Barber, 1987].

More recently it has been demonstrated that a single monoclonal antibody (MAb) specific for a short oligopeptide tag can be used to assemble multiple tag-linked antigens into SMAA complexes [Hanke *et al.*, 1992; 1994]. For example, a variety of viral proteins from

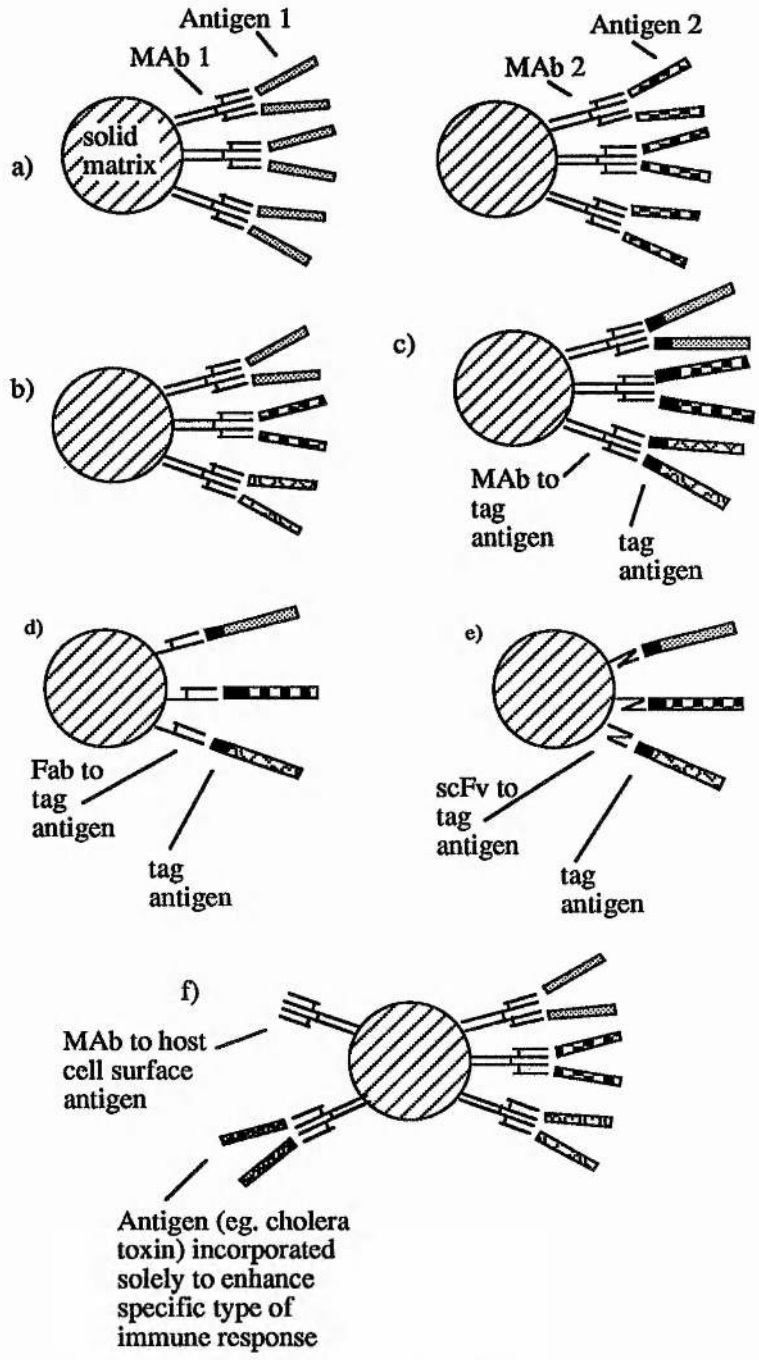


Figure 4. Solid matrix-antibody-antigen (SMAA) complexes.
Schematic illustration of the construction of solid matrix-antibody-antigen (SMAA) complexes as multivalent immunogens. (a) Different monoclonal antibodies (MAbs) are attached to independent solid matrices prior to the binding of antigen. The resulting SMAA complexes are mixed together before being used as immunogens. (b) Different MAbs are attached to the same solid matrix prior to the formation of SMAA complexes. (c) A single MAb that recognises a tag antigen, which may be aatached to different microbial antigens, is used in the construction of SMAA complexes. (d) and (e) are the same as (c) only Fab or single chain Fv fragments of antibodies respectively are used in the construction of modified SMAA complexes. (f) is the same as (b) except that the additional MAbs (e.g. to host cell surface antigens) and proteins (e.g. cholera toxin) are also attached to the solid matrix specifically to enhance particular types of immune responses (e.g. mucosal immunity)
(Taken from Randall, 1993)

Simian Immunodeficiency Virus (SIV) have been cloned and expressed, from a modified vector pQE9-Pk, in *E.coli* such that the recombinant proteins have a small histidine (His) tag at their N-termini and a small oligopeptide tag, termed Pk, at their C-termini. The Pk tag is recognised by monoclonal antibody SV5-P-k [Southern *et al.*, 1991]. These recombinant proteins can thus be purified on nickel columns, via the histidine tag, and then on immune-affinity columns by the SV5-P-k MAb. The combination of the two affinity purification steps significantly improves the purity and selects for full-size proteins. Moreover, using the MAb SV5-P-k in the second purification step, Pk-linked antigens can be assembled directly into SMAA complexes for use as vaccines [Randall *et al.*, 1993; Hanke *et al.*, 1994].

B.5. Genetic Immunisation.

Probably one of the most novel vaccination strategies to emerge in recent years, is the direct inoculation of the foreign gene(s) into living animals [Nabel *et al.*, 1990; Wolff *et al.*, 1990]. Such a process is referred to as naked DNA, or genetic, immunisation and potentially offers an extremely powerful approach for combating disease caused by viruses. One advantage is that the foreign protein is synthesised within the host cells and is thus capable of inducing $CD8^+$ CTL. For example, vaccination of experimental animals with DNA encoding the conserved internal nucleoprotein of influenza virus A, resulted in CTL-induced cross-protection from sublethal challenge with an antigenically distinct isolate of the virus [Ulmer *et al.*, 1993]. Another advantage is that the expressed protein will undertake the native conformation, an extremely important point when considering the induction of neutralising antibodies.

Naturally, as with all vaccines there are drawbacks to genetic immunisation. One of these drawbacks is the need for an efficient system that permits maximal uptake of DNA into the target cells. So far, the most common method for transfecting DNA into cells *in vivo* is direct immunisation of the genetic material into muscle cells, which are surprisingly permissive in their uptake of DNA after treatment with bupivacaine. However, other strategies are emerging. For example, inhalation of DNA incorporated into cationic liposomes, has been shown to result in the expression of the target protein in both the

airways of the epithelium and the alveolar cells lining the lungs [Stribling *et al.*, 1992]. DNA-coated gold particles can be directly inoculated into tissues of a living animal by use of a gene gun [Fynan *et al.*, 1993]. There are also several safety considerations in the use of DNA immunisation. For example, it will be important to determine if inoculated DNA integrates into the host chromosome, and whether the genetic material can induce anti-DNA antibodies that cross-react with host DNA.

B.6. Mucosal Immunisation.

The increasing evidence that priming of the mucosal system to a particular antigen can sometimes lead to immunity at other mucosal sites as well as systemic immunity, has increased research into vaccine strategies that promote immunity at mucosal surfaces. However, many strategies employed for systemic immunisations have been tried by the mucosal route with limited success. Furthermore, in general it has been extremely difficult to induce optimal antigen specific IgA responses following oral immunisation with soluble protein antigens. In part, this is due to the problem of degradation of the antigens in the acidic environment of the gut and has necessitated large amounts of antigen needing to be administered to overcome this problem. However, prolonged feeding of large doses of oral protein antigen can also lead to tolerance [Challacombe and Tomasi, 1987].

Several of the vaccine strategies described in the preceding sections have been adapted as mucosal vaccines, and are briefly outlined below. Other novel strategies that are showing particular promise as mucosal based vaccines are described in greater detail.

B.6.1. Biodegradable polymer microspheres (microcapsules).

Microspheres are biodegradable polymer matrices that can be used to encapsulate target antigens and protect the antigen from acidic and enzymatic degradation in the gastrointestinal tract. The most studied polymers are those made from lactic and glycic acids, e.g. poly lactide-co-glycolide, which are normal constituents in mammalian energy metabolism. Such spheres can vary in size depending on the constituents of the polymer matrix, and the biodegradation of the polymer is usually the rate controlling step

in the release of the antigens [Cohen *et al.*, 1991]. The release of antigen is controlled through its diffusion in the matrix pores and the speed of degradation of the matrix. Large polymers can release antigen slowly and thus have the potential to restimulate the immune system over a long period of time. It has been shown that formalin inactivated whole influenza virus A retained its immunogenicity when encapsulated in lactide co-glycolipid microspheres [Moldoveneau *et al.*, 1989] and that such microspheres induced antibody responses on the levels similar to parental immunisation with the killed vaccine. In addition, it was later found that such vaccination protected experimental animals against lethal virus challenge [Moldoveneanu *et al.*, 1993]. Microspheres have been shown to be efficiently uptaken by M cells in the Peyers patch, the hydrophobicity of the microsphere having important implications on the efficiency of this uptake. Microspheres, therefore may be used for both parental and mucosal immunisation.

However, one problem with microspheres is that during their production they are exposed to harsh, organic solvents e.g. methylene chloride, and trace amounts of this toxic compound have been found inside microspheres [Benoit *et al.*, 1986]. The presence of such substances will naturally prevent approval of microcapsules for human use. Furthermore, such harsh conditions may denature the antigen of interest, destroying important epitopes, particularly virus glycoproteins. Consequently, different, more gentle strategies are currently being developed for creating the capsules. It will be important to characterise the shelf-life of the microspheres and the ideal conditions for their storage. The possibility that the polymer will spontaneously hydrolyse and/or the encapsulated proteins aggregate in the presence of ambient moisture, remains an additional concern [Domb *et al.*, 1987].

B.6.2. Live recombinant bacterial vectors.

Live recombinant bacterial vectors e.g. attenuated Salmonella typhimurium are showing promise as novel carriers for delivery of foreign proteins to the mucosal immune system [Aggarwal *et al.*, 1990]. Many of the new strains of Salmonella that are currently being investigated as potential carriers are auxotrophs [Hoiseth and Stocker, 1981]. As

such they can only replicate in the body to a limited extent, and are potentially safe for use in immunocompromised individuals [Stocker *et al.*, 1983]. Several studies have been conducted on the efficacy of attenuated Salmonella strains as carriers for foreign viral genes. For example, incorporation of Hepatitis B virus surface protein [Wu *et al.*, 1989] and an influenza A virus haemagglutinin epitope [McEwen *et al.*, 1992] into Salmonella leads to the induction of humoral immunity to the respective viruses. One problem with Samonella as a carrier is that the insertion of foreign genes using antibiotic resistant plasmids is undesirable [Schodel *et al.*, 1994].

Recombinant BCG has also been shown to be an efficient oral vaccine delivery system [Lagranderie *et al.*, 1993].

B.6.3 Cholera toxin (CT) and *Escherichia coli* enterotoxin (LT) as mucosal adjuvants.

The gastrointestinal toxins, cholera toxin (CT) secreted from *Vibrio cholerae* and the related *E.coli* heat-labile enterotoxin (LT), which cause severe diarrhoeic disease in infected individuals, are proving promising as mucosal adjuvants for unrelated antigens co-administered at the same time as the toxins [Lycke and Holmgren, 1986; Clements, 1990]. Both CT and LT have the same basic structure, composed of two subunits; the A subunit of approximately 28kD carriers the enzymatic activity of the toxins (see below) and the B subunits, made up of 5 identical polypeptide chains (11.6kD each) arranged as a pentamer, functions to bind the toxins onto the surface of target cells via its affinity for the glycolipid, GM1-ganglioside, a normal constituent of cell membranes [Clements and Finklestein, 1979; Gill *et al.*, 1981]. In addition to GM1-ganglioside, LT (unlike CT) can also bind to certain sugars [Holmgren, 1973; 1985]. The A subunit is located in the central core of the B subunit pentamer [Sixma *et al.*, 1991; 1993], and upon binding of the toxin to the target cells, enters the cell and ADP-ribosylates the a subunit of the Gs regulatory protein [Dominguez *et al.*, 1987], which results in the Gs protein remaining in an activated state. As a consequence of this, intracellular cyclic AMP levels rise, resulting in the activation of protein kinase A, which is capable of phosphorylating the cystic fibrosis transmembrane receptor (CFTR), a

regulated chloride ion channel [reviewed by Roidan, 1993]. Phosphorylation of the CFTR causes the ion gate to open, resulting in an efflux of chloride ions (and water) into the lumen of the gut, causing characteristic symptoms of the disease. CT and LT are extremely potent mucosal immunogens, eliciting high levels of IgA antibodies to themselves following intragastric administration [Pierce and Gowans, 1975]. Of more significance for vaccine technology, both CT and LT have been shown to promote mucosal immunity to unrelated antigens administered at the same time as the toxins [Lycke and Holmgren, 1986; Elson and Ealding, 1984].The exact mechanism(s) responsible for the adjuvanticity of the toxins is unknown, but several *in vitro* and *in vivo* assays have demonstrated that they are capable of modulating a variety of immune functions, including enhancement of antigen presentation by macrophage [Bromander *et al.*, 1991], as well as promoting the switching of IgM secreting B cells, to secrete IgA [Lycke and Strober, 1989]. In addition, CT has been shown to exert both enhancing and inhibitory effects on the proliferation of B and T cells *in vitro* [Woogen *et al.*, 1987] and possibly, *in vivo*. In part, the potentiation of mucosal immunity to unrelated antigens is thought to be under control of the immune response genes [Elson and Ealding, 1987].

Naturally, the highly toxic nature of the toxins, prevents their use in man, and there have been several attempts to determine if the adjuvanticity of the toxins can be separated from the toxicity properties i.e.whether the B subunits themselves, which do not contain any ADP-ribosylating activity, contain the adjuvant function of the toxins. This has led to several research programmes analysing the ability of CTB (or LTB) to promote immunity to either co-administered antigens or antigens fused to the subunits by chemical or genetic means [Clements, 1990; Sanchez *et al.*, 1988, Klipstein, *et al.*, 1982; Schodel *et al.*, 1991]. The results from such work has been largely inconclusive in identifying the adjuvant properties of CT and LT. In some instances, the B subunits were shown to enhance the immune response [Schodel and Will, 1989; Dertzbaugh *et al* ., 1990], but in others, the presence of the A subunit was necessary to promote immunity to the target antigen [Lycke and Holmgren, 1986; Lycke *et al.*, 1992]. This discrepancy in the results has been attributed to

contamination of the B subunit preparations, (which are prepared from purified whole toxin) with minor amounts of the A subunit [Vajdy and Lycke, 1993].

More recently, the genes of the B subunits of CT and LT have been cloned and this has facilitated the construction of a number of plasmid vectors, that permit the addition of foreign sequences onto the 3' and 5'-ends of the subunits [Sandkvist *et al.*, 1987; Dertzbaugh *et al.*, 1990]. Although genetic linkage of antigens to LTB (and CTB) has obvious advantages over chemical linkage, in that the recombinant proteins are structurally defined, there are several problems to be overcome. For example, the ability of LTB and CTB to form pentamers is thought to be crucial for their immunogenicity (and hence adjuvanticity), since several studies have suggested that efficient binding of the toxins to GM1-ganglioside requires more than one B subunit [Sixma *et al.*, 1992; Iida *et al.*, 1989]. Certain amino acid additions at the C-terminus of LTB have been shown to prevent pentamerisation and subsequently association with GM1-ganglioside [Sandkvist *et al.*, 1987], although the exact amino acid restraints that lead to this are, at present, unknown. A second consideration in the use of CTB or LTB-fusion proteins is the availability of a suitable expression vector and purification protocols that can be used to purify the fusion proteins without damaging important epitopes on either the toxin subunits or the fused antigen. *E.coli* has, in the past, been employed as an expression system for LTB-fusions, but more recently, *Vibrio cholerae* species have been shown to be amenable to the expression of LTB-fusions [Schodel *et al.*, 1991]. One such vector receiving current attention is *Vibrio sp.* 60, a non-pathogenic strain of *Vibrio* [Leece and Hirst, 1992; Amin and Hirst, 1994]. The use of *Vibrio* species as vectors for LTB- and CTB-fusions, is particularly attractive, since this organism, unlike *E.coli*, can secrete oligomeric toxins (CT and LT) [Hirst *et al.*, 1984; Neill *et al.*, 1983] or their B subunits [Sandkvist *et al.*, 1987; Schodel *et al.*, 1991], across the outer membrane and into the external culture medium, where the fusion protein can be readily harvested from the culture medium. Other bacterial vectors have also been shown to be capable of expressing LTB-fusion proteins, e.g. salmonella, [Clements and Cardenas, 1993; Sanchez *et al.*, 1988] and this presents the opportunity the LTB-fusions may be delivered *in situ*, by the intestinal colonising bacteria.

C. Simian Immunodeficiency Virus (SIV).

One of the major challenges for virological and immunological research is to develop successful and practical vaccines to Human Immunodeficiency Virus (HIV). However, from work to date, it is not clear what are the parameters of protective immunity to HIV [reviewed by Levy, 1993]. Furthermore, HIV does not cause disease in any animal model system. Consequently, to try and develop vaccines against HIV, other animal model systems have been developed. The best characterised of these being infection of macaques and rhesus monkeys with Simian Immunodeficiency Virus (SIV).

SIV, like HIV, is a member of the lentivirus subfamily of retroviruses. All lentiviruses have a unique virion morphology/ morphogenesis that distinguishes them from other retroviruses. Furthermore, lentiviruses are not oncogenic but instead induce chronic, debilitating diseases following long term persistent infections. SIV has several properties that make it a useful model for HIV infection. For example, it shares biological and genetic characteristics with HIV and there is considerable sequence homology in the major structural and regulatory proteins of SIV and HIV-2 [Dersoriers *et al.*, 1988; Letvin *et al.*, 1985].

One of the key features of SIV and HIV is their ability to persist in spite of an apparently strong host response to the virus. Infected individuals may remain well for years, while maintaining easily detectable humoral and cellular immune responses, only to succumb eventually to the virus. This ability to persist and cause a slow progressing disease, seems to be unique to the lentivirus subgroup of retroviruses. Like HIV, SIV has a tropism for the CD4 molecule, thus principally infects $CD4^+$ T cells and monocytes, resulting in debilitation in their numbers leading to a chronic immunodeficiency. Some strains of SIV are capable of inducing fatal immunodeficiency disease, similar to AIDS in man, within a time scale that makes it suitable for laboratory investigations. For example, one of the most common clinical signs in macaques infected with SIV is diarrhoea, often associated with pathogenic bacteria or protozoa. The diarrhoea is often associated with a wasting disease similar to HIV in humans.

SIV infected macaques also develop immunological abnormalities including a decrease in CD4+ lymphocyte numbers [Letvin *et al.*, 1985], decreased responsiveness of peripheral blood lymphocytes to mitogenic stimulation, and thymic atrophy. Immunological abnormalities result in a range of opportunistic infections, many of which are seen in humans infected with HIV-1.

Interest in simian immunodeficiency viruses (SIVs) originates primarily from three sources. First, a common or related origin of SIV and HIV has been suggested because the viruses are similar, both biologically and at the molecular level [Coffin, 1986; Daniel *et al.*, 1985; Letvin *et al.*, 1985]. Secondly, the similarities between HIV and SIV go beyond structural features. SIVmac can be isolated from infected cells using identical methods used for isolating HIV, moreover the ability of SIV to infect human and monkey lymphocytes depends on the presence of the CD4 molecule on their surface [Kannagi *et al.*, 1985]. Thirdly, when SIV was first isolated, not only was its morphology and culture characteristics similar to HIV but also its antigenic properties indicated that it was related to HIV-1 [Kanki *et al.*, 1985]. Also, radioimmuneprecipitation analysis revealed SIV-proteins of 160, 120, 55 and 24 kilodaltons (k), all similar in size to the major gag proteins and external envelope glycoprotein of HIV.

The genomes of SIV and HIV-2 have about 75% nucleotide homology but both have only 40% of their sequences in common with HIV-1 [Charabrati *et al.*, 1987; Franchini *et al.*, 1987]. The overall organisation of their genomes are remarkably similar. The sequence of gag and pol genes are highly conserved between primate lentiviruses. The gag gene codes for a precursor polypeptide of about 55kD which, in analogy to other lentiviruses, is cleaved into polypeptides of about 17, 25 and 12 kD. There are only two amino acid differences between p27 of SIV (strain SIVmac) and HIV-2, but 13 differences between HIV-1 [Benveniste *et al.*, 1986]. The cleavage site for pol precursor protein that yields the protease and the polymerase is conserved in HIV-1, HIV-2 and SIVmac [DiMarzo *et al.*, 1986].

C.1. SIV, Protective Immunity and Vaccination.

There have been a number of studies on the induction of protective immunity to SIV [see review by Stott, 1994], and a general out-line is presented below;

(a) vaccination with attenuated viruses can protect against infection with the wild-type viruses. The basis of this protection is unknown, but recent results using SIV/HIV chimeric viruses suggest that cell-mediated immune responses, rather than neutralising antibody, are responsible for the protection seen.

(b) immunisation with whole killed cell virus vaccine can protect, but here the protection appears to be mediated via anti-host cell responses, rather than via anti-virus responses. This may be because lentiviruses incorporate host cell proteins into their envelope, e.g. class I and class II MHC molecules. Indeed, protection can be mediated by generating an anti-class II MHC response.

(c) no recombinant vaccine, either purified protein in a variety of adjuvants or expression vectors such as vaccinia virus, has been able to induce protective immunity to SIV. This is despite the finding that many recombinant vaccines that incorporate the envelope glycoprotein of SIV induce good neutralising antibodies to the virus. However, such vaccines, while failing to protect against infection, may reduce the initial virus load in infected cells.

The positive message for these results is that protective immunity can be induced by vaccination. Unfortunately, for safety considerations it may not be acceptable to use an attenuated HIV vaccine. Consequently, the challenge is to develop a novel and acceptable method of vaccination which can induce protective immunity against lentiviruses.

One thing also to bear in mind when considering the design of anti-SIV/HIV vaccine is that the natural route of infection by the virus is through mucosal surfaces. To date, the vaccine challenge studies with SIV have rarely used this method of infection and it may be necessary to induce mucosal immune responses to protect against natural infection with HIV.

D. Project Objectives.

Solid matrix-antibody-antigen complexes, as described in Section B.4.2.3.4, are showing promise as potential multivalent immunogens. Yet several problems exist in the wide-spread use of SMAA complexes as human vaccines. For example, a suitable biodegradable solid matrix that is readily acceptable for use in man, needs to be developed. At present, alum is being employed as the solid matrix [Randall *et al.*, 1993; 1994], but several problems with the use of alum have been described previously. However, the advancement in SMAA complex technology has opened the door for new strategies that could over-ride the need for the solid matrix. For example, it might be possible to generate highly immunogenic complexes by joining a tag-linked adjuvant to one arm of an anti-tag antibody, and tag-linked antigen to the other arm. This project has been concerned with such a strategy. LTB has been used as the adjuvant and antigen-antibody-LTB complexes have been constructed. The use of LTB as the adjuvant in the project, gave the added bonus that it increased the chances of such complexes acting as mucosal immunogens.

In this project, a small epitope tag (termed Pk) was attached to LTB and SIV antigens, that were also tagged with the Pk epitope, were linked to LTB via an antibody (anti-Pk) bridge. The systemic and mucosal responses to such complexes were evaluated.

MATERIALS AND METHODS.

1. Recombinant DNA techniques.

1.1. Plasmids, bacterial strains and culture conditions.

The bacterial strains employed in this project were *E.coli* strain HB101 (Bolivar *et al.*, 1977) and *Vibrio sp.*60 (Ichige *et al.*, 1988). Liquid cultures of *E.coli* were grown in either in Luria broth (L-broth) [10g/l bactotryptone (Difco), 5 g/l yeast extract (Difco), 10mM NaCl, pH 7.5] or 2X TYE broth (16g/l bacto-tryptone, 10g/l yeast extract, 20mM NaCl, pH 7.5) supplemented with 100µg/ml ampicillin (Sigma). *Vibrio sp.* 60 is a non-pathogenic marine *Vibrio*, recently described by Ichige *et al.*, [1988]. Liquid cultures of *Vibrio sp.* 60 were grown in L-broth supplemented with an additional 2% NaCl and 100µg/ml ampicillin.

The vector pMMB66, a derivative of the broad host range plasmid, RSF1010, was used to clone the gene encoding the B subunit of *E.coli* heat-labile enterotoxin subunit B, which has a 21 amino acid signal sequence followed by 103 amino acid mature sequence [Sandkvist *et al.*, 1987]. Upstream of the B subunit gene is a *tac* promotor. The 3'-end of the gene was modified as described in Sandkvist *et al.*, [1987], to give plasmid pMMB138, which resulted in a B subunit with a short carboxyl-terminal extension and a HindIII site near the end of the gene. Into this site an oligonucleotide (see fig. 5) was inserted, to give plasmid pTRH101R. Plasmid pTRH101R was a kind gift from Dr. T.R. Hirst, University of Kent.

1.2. Synthesis of synthetic oligonucleotides.

Synthetic oligonucleotides were made using an Applied Biosystems 381A DNA synthesiser (kindly performed by I. Armitt). The oligonuucleotides were resuspended in 200µl of sterile water and precipitated with 1/10th volume of 3M sodium acetate, 1/100th volume 1M magnesium acetate and 3 volumes absolute ethanol. The precipitate was pelleted by high speed centrifugation at 4°C for 15 minutes in a bench top microfuge and then

pTRH101R

102 103
...glu lys Le(u Leu Glu Asp Pro Leu Val Pro Ala Leu Arg Thr G)ln Leu .....
..GAA AAG CT(T CTA GAG GAT CCA CTA GTG CCG GCC CTG CGG ACC C)AG CTT.....

HindIII Bam HI NaeI
XbaI SpeI

Figure 5. Plasmid pTHR101R.

The oligonucleotide (bracketed) was inserted into the HindIII site of pMMB138. The residue numbers 102, 103 104 etc, refer to those found in the protein EtxB138 expressed from pMMB138 [Sandkvist *et al.*, 1987]. The underlined areas represent the sequences recognised by the named endonuclease restriction enzymes.

positive stranded oligonucleotide:

5' AGCTGGGAAAGCCGATCCCAAACCCTTTGCTGGGATTGGACTCCACCTAGA 3'

negative stranded oligonucleotide:

5' CTAGTCTAGGTGGAGTCCAATCCCAGCAAAGGGTTTGGGATCGGCTTTCC 3'

Figure 6. Sequence of the positive and negative oligonucleotides.

The figure shows the oligonucleotide linkers that were inserted between the HindIII and SpeI endonuclease restriction sites of plasmid pTRH101R.

resuspended in 200µl of sterile water. The DNA concentration of the oligonucleotides was determined by measuring the UV absorbance at 260nm (an absorbance of 1.0 at 260nm is taken to be the equivalent to 40µg/ml).

The sequences of the positive and negative oligonucleotides are given Fig. 6. The oligonucleotides were annealed by mixing 50µl of the positive stranded olignucleotide with 50µl of the negative stranded oligonucleotide, boiling for 2 minutes at 100°C, then leaving the oligonucleotides to anneal at room temperature for 30 minutes. Once annealed, the oligonucleotides were 5'-phosphorylated using T4 polynucleotide kinase (New England BioLabs) by mixing 100µl of the annealed oligonucleotides with 11µl of 10X ligation buffer (bacteriophage T4 ligation buffer) and 2µl T4 polynucleotide kinase. The reaction was performed at 37°C for 30 minutes. The 5'-phosphorylated double-stranded oligonucleotides were then ethanol precipitated and resuspended in 50µl of distilled water.

1.3. Construction of recombinant plasmid pTRH-Pk.

The basic cloning procedures involved in the construction of plasmid pTRH-Pk are modifications of standard protocols from Maniatis *et al.*, 1982.

1.3.1. Preparation of plasmid DNA.

A 10µl sample of *E.coli* containing plasmid pTRH101R was used to inoculate a 10 ml volume of 2 x TYE broth supplemented with 100µg/ml ampicillin (TYE/AMP) and the culture was incubated for 18 hours at 37°C, on an orbital shaker. 100 ml of TYE/AMP was inoculated with a 1 ml sample of the overnight culture and following incubation at 37°C for 16 hours, the bacteria were harvested by centrifugation at 12,000g, at room temperature for 10 minutes. The desired plasmid was then isolated using a modification of the alkali lysis protocol described by Birnboim and Doly [1979]. The bacterial pellets were washed once by resuspending the pellets in 10 ml of solution I (25mM Tris-HCl, pH 8.0, 50mM Glucose, 10mM EDTA) then centrifugating the suspension at 12,000g for 10 minutes at room temperature. The supernatant was discarded and the bacterial pellets resuspended in 4 ml of solution I supplemented with 10mg/ml lysozyme and 30µl of DNase-free RNase (Boehringer

Mannhein), and during a 30 minute incubation at room temperature, the suspension was vortexed vigorously every five minutes. To the suspension, 8 ml of freshly prepared solution II [0.2M NaCl, 1% (w/v) SDS] was added, and after a ten minute incubation at room temperature, a further 6 ml of solution K (5M potassium acetate, 11.5% (v/v) glacial acetic acid, pH 5.5) was added and the suspension incubated for a further 5 minutes at room temperature. The suspension was centrifuged at 12,000g for 10 minutes at room temperature. The resulting supernatant was carefully removed and filtered through two layers of cheese-cloth into a clean beaker. The plasmid DNA was precipitated by measuring the volume of the filtrate then adding 0.6 volumes of isopropanol, vortexing vigorously, followed by centrifugation at 4°C for 10 minutes at 12,000g. The supernatant was discarded and the pelleted plasmid DNA dried *in vacuo*. The dried pellet was resuspended in 5 ml of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), following which, 10µl of DNase-free RNase was added, and the suspension incubated at 37°C for 30 minutes. The plasmid DNA was then purified by phenol/chloroform extraction. The DNA solution was mixed with an equal volume of phenol, vortexed vigorously, and centrifuged at 12,000g for 10 minutes at room temperature. The aqueous solution, containing plasmid DNA, was gently removed, placed in a fresh tube and the volume measured. To this aqueous solution, an equal volume of 1:1 ratio of phenol:chloroform was added, the solution vortexed vigorously and centrifuged as before. The aqueous solution was again retained, measured and the contaminating phenol removed by adding 1 volume of chloroform, vortexing vigorously, then centrifugating at 12,000g for 10 minutes at room temperature. After removal of the aqueous layer to a fresh tube, the plasmid DNA was ethanol precipitated. The volume of the aqueous layer was measured and 2.5 volumes of ethanol added, followed by a 1/10th volume 3M sodium acetate. The plasmid DNA was then pelleted by centrifugation at 12,000g for 10 minutes at 4°C. The precipitated plasmid DNA was gently washed with 70% ethanol, excess alcohol removed by inverting the tube over a tissue, and then dried *in vacuo*. Once dried, the plasmid DNA was resuspended in 200µl of sterile water, a 20µl sample removed to determine the concentration and purity of the plasmid DNA preparation (see below), and the remaining volume stored at -20°C until needed.

1.3.2. Estimation of the concentration and purity of DNA in the plasmid preparation.

The concentration of the DNA in the plasmid preparation was determined by measuring the absorbance at 260nm of a 20µl sample plasmid DNA (diluted 1:100 in TE buffer) using a spectrometer. By using the formula that 1 unit of absorbance is equivalent to 50µg/ml double stranded DNA, the concentration of the plasmid could be estimated.

Purity of the plasmid DNA preparation was estimated by measuring the absorbance at 260 nm and 280 nm, and determining the ratio between the two readings. Values of 1.8 (260nm:280nm) were taken to represent highly purified DNA.

1.3.3. Cleavage of plasmid DNA

Restriction endonucleases Hind III and Spe I (both from New England BioLabs) used in the cleavage of the plasmid DNA were supplied with their appropriate 10X buffer. The concentrations of enzymes employed in the digestion were chosen following manufacturer's recommendations.

A 20µl sample (approximately 2µg) of purified DNA was mixed with 10µl (12 Units) Spe I, 5µl (20 Units) Hind III, 20µl 10 X buffer, 2µl acetylated-bovine serum albumin (BSA), the volume made up to 200µl with distilled water and the digestion was allowed to proceed for 18 hours at 37°C. Following examination of the enzyme digested plasmid by horizontal agarose gel electrophoresis (below) to establish that the cleavage had been successful, the ends of the cleaved plasmid were dephosphorylated by the addition of 0.5µl (12U) of alkaline phosphatase (Boehringer Mannheim) to the DNA solution, followed by a one hour incubation at 37°C. The alkaline phosphatase was inactivated by adding EGTA to a final concentration of 20mM and incubating the solution at 65°C for 10 minutes.

1.3.4. Horizontal agarose gel electrophoresis.

The digested plasmid DNA was examined by agarose-gel electrophoresis, in an agarose horizontal minigel apparatus (Pharmacia), to determine whether the cleavage had been successful. A 2µl sample of digested DNA was separated on a 10 x 10 x 0.5cm agarose gel

containing 1% agarose (Sigma) reconstituted in TBE buffer (90mM Tris-borate, 2mM EDTA pH 8.0). The cleaved plasmid was mixed 1:5 (v/v) with 6X loading buffer [0.25% (w/v) bromophenol blue, 30% (v/v) glycerol], added to the gel and horizontally electrophoresed at 100 volts until the dye front migrated three-quarters of the way through the gel. As a size marker, phage lambda DNA, cleaved with restriction enzyme Hind III (Boehringer Mannheim), was similarly electrophoresed. The gel was then stained with 1µg/ml ethidium bromide in TBE buffer and the DNA bands visualized on an ultra violet transilluminator.

1.3.5. Purification of cleaved plasmid by electroelution.

After ascertaining that the plasmid had been successfully cleaved, the cut plasmid was electroeluted by the following procedure. Plasmid DNA was cut as before and the DNA bands were separated by electrophoresis through a 1% horizontal agarose-gel. Once the dye front migrated three-quarters of the way through the gel, the gel was removed from the rig and placed on a UV transilluminator to visualize the location of the cut plasmid. A 1mm wide trough was carefully excised just in front of the migrating DNA band and the gel returned to the rig. TBE buffer was gently poured into the electrode tanks making sure it did not overflow the surface of the gel, and using a micropipette, the excised trough was filled with TBE buffer. The gel was electrophoresed for 30 seconds at 100 volts, after which, the power was disconnected. The fluid in the excised trough was extracted with a micropipette and placed in a clean eppendorf vial, the trough was then re-filled with fresh TBE buffer. This electroelution process was repeated 7-9 times, the progress of which was monitored on the UV illuminator, to determine no DNA remained within the gel. The DNA-containing buffer aliquots were pooled, the DNA ethanol precipitated and dried, as described above. For vector fragments, the DNA was incubated with alkaline phosphatase (Boehringer Mannheim) for 1 hour at 37°C prior to phenol/chloroform extraction in order to prevent recirculization during subsequent ligation reactions. Dried DNA was resuspended in 20µl of water and a 2µl sample used to estimate the level of DNA recovery by 1% horizontal agarose-gel electrophoresis.

1.3.6. DNA ligation.

Approximately 400ng of vector DNA and 40-400ng of the double stranded oligonucleotide insert were combined in a total of 20µl of ligation buffer (50mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25mg/ml BSA) containing 200U T4 DNA ligase (New England BioLabs) and incubated at 16°C for 1 hour. The ligation mixture was used directly for the transformation of competent *E.coli.*

1.4. Preparation and transformation of competent bacteria.

E. coli strain JM101 was made competent using the modified rubidium chloride (RbCl) method of Kushner (1978). Briefly, a 60 ml volume of 2 x TYE broth was inoculated with *E.coli* strain JM101 and the bacteria incubated at 37°C until the OD_{600} reached approximately 0.5. The bacteria were then cooled by incubating the culture flask on ice for 10 minutes, and then harvested by centrifugation at 12,000g for 10 minutes at 4°C. The bacterial pellet was resuspended in a 20 ml volume of ice-cold MOPS I solution (1M MOPS pH 7.0, 100mM rubidium chloride), centrifuged as above, and then resuspended in a 20 ml volume of ice-cold MOPS II solution (1M MOPS pH 6.5, 700mM $CaCl_2$, 100mM rubidium chloride). After centrifugation at 12,000g for 10 minutes at 4°C, the pelleted bacteria were resuspended in a 2 ml volume of MOPS II solution. Competent bacteria were either used directly for DNA transformation or stored at 4°C for one week.

Transformation of the bacteria was achieved by combining one-half of the 20µl ligation reaction with 100-200µl of competent bacteria and incubating on ice for 30 minutes, with occasional end-over-end shaking. The bacteria were then heat-shocked at 42°C for 2.5 minutes to promote DNA uptake, returned to ice for 5 minutes, and then transferred into a 1ml volume of 2 x TYE broth. The bacteria were incubated for one hour at 37°C without antibiotics, under constant agitation, following which ampicillin-resistant recombinants were selected after growth on Luria-agar (L-agar) plates (below) supplemented with 100 µg/ml ampicillin, for 24 hours at 37°C.

1.5. Preparation of L-agar plates.

100ml of L-agar (10g/l Tryptone, 5g/l yeast extract, 10g/l NaCl, NaOH, pH 7.5, 2% agar) was placed in a water bath at 100°C. When all the agar had melted, the solution was left to cool to approximately 50°C and then supplemented with 100µg/ml ampicillin. The agar solution was immediately poured into petri-dishes (Sterilin) and allowed to set. Once set, the plates were dried at 42°C and sterilized by ultraviolet radiation. The prepared plates were then stored at 4°C in sealed polyethylene bags, until use.

1.6. Screening for pTRH-Pk recombinants.

Single colonies growing on antibiotic supplemented L-agar plates were selected and used to inoculate separate 10 ml cultures of Luria-broth containing 100 µg/ml ampicillin. The bacteria were then cultured for 18 hours at 37°C on an orbital shaker. A 100µl sample of the overnight culture was used to inoculate a 2 ml volume of antibiotic-supplemented L-broth solution and the bacteria grown at 37°C until the OD_{600} reached 0.4. At this point, IPTG was added to each culture to give a final concentration of 0.5mM and the incubation allowed to continue for 4 hours at 37°C. The bacteria were harvested and a 1 ml sample removed, centrifuged at high speed in a bench-top microfuge and the bacterial pellets resuspended in a 1ml volume of ice-cold 0.25M sucrose, 0.1M sodium phosphate buffer, pH 7.6, to which was added 20µg/ml lysozyme and 5mM EDTA. The solution was incubated on ice for 10 minutes, sonicated at full power for 4 seconds using a Soniprep 150 sonicator, then centrifuged at high speed in a bench top centrifuge for 5 minutes at room temperature and the supernatant retained.

Bacterial clones producing LTB-Pk were determined by dot-blot assay. A 10µl sample from each of the cell lysates was added, in duplicate, to the wells of a terasaki plate (Sterilin) and the plates were then electrophoreseed with a 5 cm x 7.5 cm nitrocellulose filter (Gelman Sciences). A second terasaki plate was placed on top of the nitrocellulose filter, making sure the wells of each plate were aligned, and after clipping the plates tightly together, the plates were inverted to bring the lysates into close contact with the nitrocellulose filter. The plates were incubated at room temperature for one hour, following which the nitrocellulose

filter was removed, and rinsed three times in phosphate buffered saline (PBS) containing 1% semi-skimmed milk. The nitrocellulose was then incubated for one hour in 20 ml of PBS supplemented with 20% semi-skimmed milk, following which the nitrocellulose filter was rinsed three times in successive 20 ml volumes of PBS containing 1% semi-skimmed milk. The blots were probed for the presence of the Pk determinant by incubating the nitrocellulose filters with a 20 ml volume of PBS supplemented with 1% semi-skimmed milk and 1: 20 dilution of anti-Pk MAb SV5-P-k (see below). After a one hour incubation at room temperature, the nitrocellulose filter was rinsed as before, then incubated with a 20 ml volume of PBS containing 1% semi-skimmed milk and a 1: 1000 dilution of ^{125}Iodine-labelled Protein A (Amersham), for a further hour at room temperature. Excess radio-label was discarded and the nitrocellulose filter washed extensively in PBS, blotted dry and then exposed to Fuji X-ray film for 18 hours at -20°C. Positive-LTB-Pk expressing clones were visualized following development of the film in a Kodak developer.

Alternatively, colonies expressing LTB-Pk following IPTG induction were screened by analysing the cellular lysates for LTB-Pk by Western blot assay.

1.7. Storage of colonies.

Bacterial colonies that were shown to express LTB-Pk were used to inoculate separate 10ml cultures of ampicillin-supplemented L-broth. The bacteria were cultured overnight at 37°C on an orbital shaker, and the following day, 0.85ml of bacterial culture was transferred to a sterile vial containing 0.15ml of sterile glycerol. The bacteria were then stored at -70°C.

2. ANTIBODIES.

Monoclonal antibody SV5-P-k (MAb SV5-P-k) recognizes an epitope on the simian virus 5 (SV5) phospho (P) and V proteins [Randall *et al.*, 1987] and it's specificity was determined in our laboratory [Southern *et al.*, 1991]. MAb SV5-P-k was purified either from tissue culture medium, or ascitic fluid stocks by affinity chromatography techniques (see below). Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) was

obtained from the Scottish Antibody Production Unit (SABU). HRP-conjugated Protein A which specifically interacts with the Fc region of murine IgG_{2a}, IgG_{2b}, and IgG_3 but not IgG_1 antibodies, was obtained from Amersham International. Goat anti-mouse IgG, IgM and IgA antibodies and alkaline-phophatase-conjugated anti-goat antibody were obtained from Sigma. Fluorescein isothiocyanate (FITC)-labelled anti-murine CD4, CD8 and Ig antibodies were supplied by Sera-Lab.

2.1. Purification of MAb SV5-P-k by affinity chromatography techniques.

MAb SV5-P-k was purified from ascitic fluid or tissue culture medium by affinity chromatography techniques using a Protein A (Pr.A)-sepharose support (Sigma). A 1ml sample of Pr.A-sepharose beads were loaded into a column and washed with 20ml of phosphate buffered saline (PBS). The resin was incubated with MAb SV5-P-k-containing fluid for one hour at room temperature, then material that failed to bind to the column was removed by washing the column in 100 column volumes of PBS. Bound MAb SV5-P-k was eluted by the addition of a buffer composed of 0.2M glycine, 0.1M NaCl, pH 2.8. 1ml fractions were collected throughout the elution process, and fractions contained purified MAb SV5-P-k were pooled and dialysed against 4 litres of PBS for 18 hours, changing the buffer once. The purified antibody was filter sterilized through a 0.22µm nylon filter (Gelman Sciences) and stored in 1 ml aliquots at 4°C until use.

3. SDS-PAGE and Western blotting.

3.1. Preparation of polyacrylamide gels.

All the polyacrylamide gels employed in this project, contained 15% acrylamide. A 15% SDS-polyacrylamide separation gel was constructed by mixing together 2.675 ml of solution A (28% Acrylamide/ 0.735% DATD), 0.625 ml of solution B (3M Tris-HCl, pH 8.5), 1.25 ml of freshly prepared solution C (0.14% ammonium persulphate), 0.425 ml distilled H_20, 25µl 20% SDS and 2.5µl N,N,N',N'-tetra-methylethylenediamine (TEMED; Bio-Rad). Immediately after the addition of the TEMED, the gel solution was poured between the glass gel cassettes of a Bio-rad Mini Protein System gel rig, leaving a 2 cm gap at the top

of the cassette. The separation gel was carefully overlaid with 200µl of distilled water, to create an even interface, and the gel was left to polymerise at room temperature for 30 minutes. A stacking gel was prepared by mixing together 1.6 ml of solution C, 400µl of solution A, 300µl of solution D (1M Tris-HCl, pH 7.0, SDS) and 1µl TEMED. The H_20 was gently removed by inverting the gel cassette over a paper tissue, the stacking gel poured on top of the separation gel and a comb inserted to create wells. The gel was then left to polymerise at room temperature for one-two hours.

3.2 Preparation of samples for SDS-PAGE and electrophoresis.

15µl samples of protein (unless otherwise stated in the Results) were mixed with 5µl of 4x disruption buffer (25% β-mercaptoethanol, 10% SDS, 25% solution B, 12.5% glycerol) and boiled for 5 minutes at 100°C (unless otherwise stated). The disrupted protein samples were then loaded into the wells of the prepared SDS-polyacrylamide gel using a micropipette and electrophoresed in electrode buffer (0.19M Glycine, 0.025M Tris-HCl, 0.1% SDS, pH 8.0) at a constant voltage of 150 volts. When the dye front migrated to a position 0.5cm from the bottom of the gel, the current was disconnected and the gel removed from the gel rig. Thereafter, the denatured polypeptide chains were either stained with Coomassie blue stain or electroblotted onto nitrocellulose filters and analysed by Western blotting.

3.3. Coomassie blue staining.

Individual polypeptide chains were visualized by incubating the SDS-polyacrylamide gel for 20 minutes in approximately 30 ml of Coomassie blue stain solution (0.2% Coomassie brilliant blue R250 (BDH), 20% methanol and 10% acetic acid). On completion of the incubation period, the excess stain was poured off and the gel destained with repeated washing in destaining solution (10% acetic acid, 10% methanol). During both the staining and destaining steps, the gel was gently agitated on a rotatest shaker, to permit uniform staining/destaining. The gel was then either dried onto 3MM Whatman filter paper using a Bio-rad 583 vacuum gel drier for 2 hours at 80°C, or stored in sealed plastic bags.

3.4. Western blotting and autoradiography.

Polypeptide chains separated on 15% polyacrylamide gels were electroblotted onto nitrocellulose using a LKB semi dry electroblotter. Both the bottom (anode) and top (cathode) graphite plates were washed in distilled H_20 prior to electroblotting.

Four sheets of 3MM Whatman filter paper, cut to the same size as the gel, were soaked in Western Blotting (WB) buffer (20% methanol, 20mM Tris) and placed on the bottom graphite plate. A sheet of nitrocellulose, similar in size to the gel, that had been moistened in WB buffer, was placed on top of the filter papers and electrophoreseed with the polyacrylamide gel. Four more sheets of filter paper were soaked in WB buffer and layered over the gel to complete the sandwich. The top graphite plate was put in place and the electrodes attached. The gel was blotted for one hour using a current of 1mA per cm^2 of the sandwich, after which, the current was switched off and the nitrocellulose sheet removed. The nitrocellulose sheet was placed in a petri-dish (Sterilin) containing 20 ml of blocking buffer (5% semi-skimmed milk, 20mM Tris-HCl, pH 7.6, 140mM NaCl, 0.1% Tween 20) and blocked for 15 minutes at room temperature on a rotatest shaker. The nitrocellulose sheet was then rinsed three times in successive 30ml volumes of PBS, followed by a further 10 minute incubation in blocking buffer at room temperature. After rinsing in PBS as before, the nitrocellulose sheet was incubated in 20ml of blocking buffer containing a 1:1000 dilution of MAb SV5-P-k, for 30 minutes at room temperature. The nitrocellulose sheet was then rinsed in PBS and after washing twice with blocking buffer, incubated for 30 minutes in 20ml of blocking buffer containing a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated Protein A (Amersham), at room temperature. On completion of the incubation period, the nitrocellulose sheet was rinsed in PBS, then twice in the blocking buffer. After a final rinse in PBS to remove excess blocking solution, the nitrocellulose sheet was dried briefly between two 3MM Whatman filters, and transferred into a fresh petri dish containing 5 ml of enhanced chemiluminescence (ECL) Western blotting reagents (Amersham) that had been mixed according to the manufacturers recommendations. The nitrocellulose sheet was incubated in ECL reagents for 1-2 minutes, placed inside a plastic bag, then exposed to Fuji X-ray film

adjacent to an intensifying screen for 0.5-3 minutes. The film was then developed using an automatic developer (Kodak).

4. GM1-enzyme-linked immunoasorbent (GM1-ELISA) assay.

The assay out-lined below is an adaption of the procedure described by Svennerholm and Holmgren [1978]. Flat bottomed, 96-well microtitre plates (Corning) were coated with 100µl per well of 1.5µg/ml monosialioganglioside-GM1 (GM1; Sigma) dissolved in PBS, for 18 hours at 4°C. The plates were washed twice in PBS and the remaining protein-binding sites blocked in 200µl of PBS containing 1% (w/v) BSA (Sigma) at 37°C for one hour. After blocking, the plates were washed three times in PBS. Test samples were serially diluted in PBS supplemented with 0.1% (w/v) BSA (PBS/BSA) and added to the wells in a volume of 100µl and the plates were incubated at room temperature for one hour. The plates were then washed three times in PBS, and a 100µl sample of anti-Pk MAb SV5-P-k (tissue culture fluid) diluted 1:20 in PBS/BSA, was added to each well. After incubation for one hour at room temperature, the plates were washed three times in PBS containing 0.05% Tween 20 (PBS/Tween), and then 100µl of horse-radish peroxidase (HRP)-conjugated Protein A, diluted 1:1000 in PBS/BSA, was added to each well for one hour at room temperature. The plates were washed three times in PBS/Tween. A 100µl volume of α-phenylene-di-amine (1mg/ml) (Sigma) in 0.1M citrate buffer pH 4.5 (2.1g citric acid, 2.84g Na_2HPO_4 made up to 400ml with distilled water), (activated by the addition of 0.4µl/ml 30% (v/v) hydrogen peroxide) was added to each well to detect bound conjugated antibody . The plates were then incubated at room temperature for 15-30 minutes following which the adsorbance at 450nm was measured using a Dynatech MR5000 microtitre plate reader.

5. Immune-precipitation assays.

A 30µl sample of a heat-killed suspension of *Staphylococcus aureus (St. aureus)* Cowan A strain (10% w/v) was placed in a 500µl eppendorf and centrifuged at high speed in a microfuge for 5 minutes at room temperature. The supernatant was gently aspirated using a pipette tip attached to a vacuum, and the pellet resuspended in 30µl of ice-cold immune

precipitation buffer (0.65M NaCl, 20mM Tris-HCl pH 7.8, 1mM EDTA, 0.5% (v/v) NP-40, and 0.1% (w/v) azide). After centrifugation as before, the supernatant was again removed by aspiration and the *St. aureus* pellet resuspended in 30µl of PBS containing approximately 2mg/ml purified anti-Pk MAb SV5-P-k. The suspension was incubated on ice for 30 minutes to allow maximum adsorption of the MAb to the bacterium, then centrifuged at low speed in a microfuge, for 3 minutes at room temperature. The supernatant containing unbound antibody was gently removed by aspiration, taking care not to disturb the *St. aureus*-MAb pellet. To be sure no free MAb remained, the pellet was resuspended in 30µl of ice-cold immune-precipitation buffer, centrifuged as before and the supernatant gently removed. This washing step was repeated three times. The pellet was resuspended in a 15µl volume (unless otherwise stated in the Results) of the test solution and the suspension incubated on ice for one hour. After low speed centrifugation in a microfuge, the supernatant containing unprecipitated proteins was gently transferred to a fresh eppendorf vial using a micropipette, a 5µl volume of 4X disruption buffer added and the sample stored at -20°C until analysed. The precipitate was then resuspended in 30µl of ice-cold immune-precipitation buffer, centrifuged as before, and the supernatant discarded. This washing step was repeated four times to ascertain no unprecipitated proteins remained. Finally, the pellet was resuspended in 30µl of 1X disruption buffer and stored at -20°C until analysed.

6. Concentration of LTB-Pk

6.1. Concentration of LTB-Pk by ammonium sulphate precipitation.

Small scale ammonium sulphate precipitations were performed at room temperature. A one litre culture of *Vibrio sp.*60 harbouring the plasmid pTRH-Pk was maximally induced with 0.05mM IPTG for 6 hours at 30°C, and the culture supernatant separated from the bacteria by centrifugation at 12,000g for 20 minutes at 20°C. To a 200ml volume of the supernatant (containing approximately 6mg/l of crude LTB-Pk), 62.6g of solid ammonium sulphate was added very slowly with constant stirring, to give a final salt saturation level of 50%. Protein precipitation was allowed to continue for one hour, after which the solution was centrifuged at 2,500g at 4°C. The supernatant was discarded and the pellet containing

precipitated protein, was resuspended in 20ml of sterile PBS. The suspension was centrifuged at 2,500g for 20 minutes at 4°C, to pellet insoluble material. The supernatant was gently removed using a pasteur pipette and transferred to a dialysis membrane. Excess salt was removed by extensively dialysing the solution over a four day period, against 4 litres of PBS, changing the buffer twice daily. On completion of the dialysis period, the protein solution was centrifuged at 2,500g for 20 minutes at 4°C to pellet any insoluble material. The supernatant was recovered, sterilized by filtration through a 0.22μm sterile filter and stored at 4°C in 1ml aliquots until use.

6.2. Concentration of LTB-Pk by ultrafiltration and in situ dialysis.

Large volumes of culture medium (≤ 4 litres) containing crude LTB-Pk were concentrated by ultrafiltration using a GF 15 kidney dialysis membrane (Gambro®) with a porosity of 10K. Prior to use, the membrane was washed extensively in 1 litre of distilled water to remove the ethanol preservative. A 4 litre volume of culture medium containing approximately 6mg/l of LTB-Pk, was passed through two 3MM Whatman filters, and then applied to the membrane. The solution was constantly recycled through the membrane and concentrated by applying a pressure of 5-10 lbs/in^2 [see Fig. 7(a)]. This strategy helped to remove small molecules and water from the recirculating material. The solution was concentrated to 150-200ml, then dialysed *in situ* against either 20mM Tris-HCl, 10mM EDTA, pH 7.5 or 25mM Na_2PO_4, 10mM EDTA, pH 7.0 (depending on whether LTB-Pk was to be purified by hydrophobic or ion-exchange chromatography techniques, respectively), by recirculating the dialysis buffer counter-current to LTB-Pk containing solution [see Fig. 7(b)]. On completion of the dialysis period, the LTB-Pk-containing solution was recovered from the membrane and either applied directly to the appropriate chromatography column or stored at 4°C, in the presence of 0.01% sodium azide, until use.

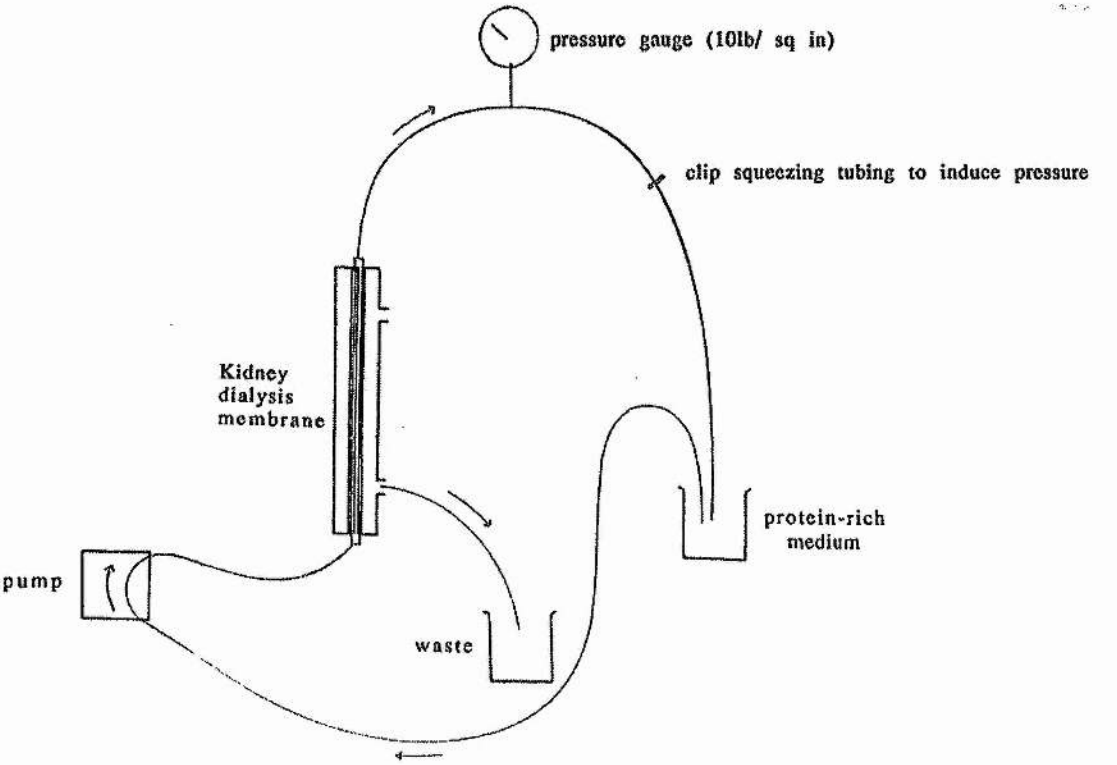
Figure 7. Concentration of LTB-Pk from *Vibrio sp.* 60 cultures by ultrafiltration.
The figure shows the GF15 kidney dialysis membrane used for the concentration and *in situ* dialysis of LTB-Pk.

Panel (a): the *Vibrio sp.*60 culture supernatant containing LTB-Pk is pumped through the dailysis unit under a pressure of 5-10lbs/in^2. The arrows indicate the direction of the flow of the medium through the membrane. This stage helped concentrate the culture medium.

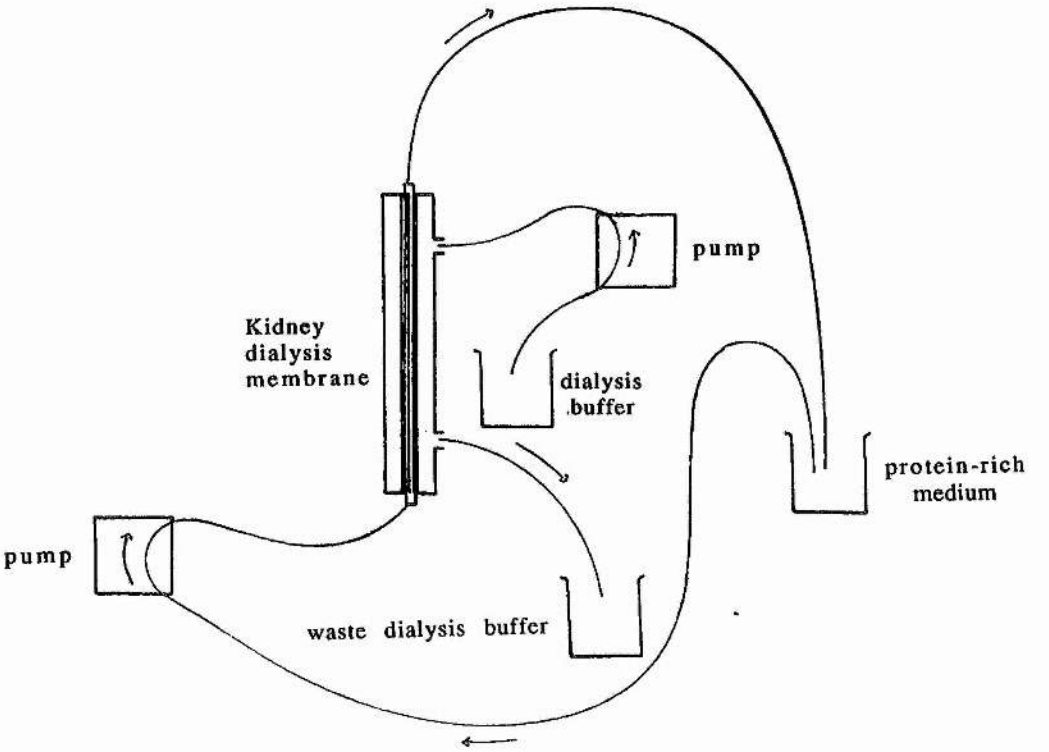
Panel (b): the concentrated material was then passed through the membrane system counter-current to the appropriate dialysis buffer (see arrows for the movement of the culture medium versus the dialysis buffer). This latter step, resulted in *in situ* dialysis of the culture medium.

(pictures courtesy of Dr. C. Botting)

Panel (a)



Panel (b)



7. Purification of LTB-Pk

7.1. Purification of LTB-Pk by Hydrophobic interaction chromatography using a phenyl superose matrix on an Fast Protein Liquid Chromatography (FPLC) System.

The procedure outlined below is a modification of the procedure described by Amin and Hirst [1994]. A 20 ml volume of culture medium containing LTB-Pk that had been concentrated by ultrafiltration and dialysed against 20mM Tris-HCl, 10mM EDTA, pH 7.5 (Section 7.2) was mixed with ammonium sulphate to a final salt saturation of 30%. The solution was slowly stirred for 1 hour at room temperature, then clarified by centrifugation at 17,400g for 20 minutes at room temperature. The resulting supernatant was retained, 5ml of which was filtered through a 0.22μm nylon filter (Gelman Sciences) and the filtrate was loaded directly onto a 1 ml FPLC Phenyl superose HR5/5 column (Pharmacia), that had been equilibrated with 20mM Tris-HCl, 10mM EDTA containing 1M $(NH_4)_2SO_4$, pH 7.5. Once the sample was loaded onto the column, a decreasing salt gradient of 1M ammonium sulphate in 20mM Tris-HCl, 10mM EDTA, pH 7.5 was applied. 1ml fractions were collected throughout the purification, and the adsorbance measured at 280nm. The fractions representing the column void were pooled and dialysed overnight against 4 litres of PBS containing 10mM EDTA at 4°C. Similarly, the fractions representative of eluted protein, were pooled and dialysed. Both samples were filter sterilized by passage through a 0.22μm sterile nylon filter and stored at 4°C until use.

7.2. Purification of LTB-Pk by hydrophobic chromatography using a Bio-rex Macroprep® t-butyl support.

The protocol for the purification of proteins by the Macroprep.® t-butyl column (Bio-rad) was based on the manufacturer's recommendations. A 5ml hydrophobic t-butyl matrix was placed in a 10ml disposable column and washed in 500ml of 20mM Tris-HCl, pH 7.5, 10mM EDTA, 1M $(NH_4)_2SO_4$. A 40ml sample of LTB-Pk prepared as described in Section 7.1, by mixing the sample with ammonium sulphate to a saturation level of 30%, then clarifying the solution by centrifugation. The prepared sample was then filtered as above and

applied directly to the equilibrated column. When all the sample had been applied, the column was washed in 20 column volumes of 20mM Tris-HCl, pH 7.5, 10mM EDTA, 1M ammonium sulphate to remove material that failed to bind to the hydrophobic matrix. Once the column had been washed the following decreasing salt solutions were applied to the column:

Volume (ml)	Salt Molarity.
200	0.75M ammonium sulphate
200	0.50M ammonium sulphate
200	0.25M ammonium sulphate

5ml fractions were collected throughout the purification, and 1µl of each fraction spotted onto nitrocellulose paper. Proteinaceous material was then detected by incubating the nitrocellulose in approximately 10ml of Naphthlene black stain (0.2% Naphthlene black, 20% methanol, 10% acetic acid) for 15 seconds, followed by destaining in a buffer composed of 10% acetic acid, 10% methanol until the background was sufficiently destained. Fractions containing maximal levels of protein (as determined by the amount of stain adsorbed by the protein spots) from the column void, were pooled and dialysed against PBS, 10mM EDTA for 18 hours at 4°C. The fractions containing eluted protein were similarly treated. After dialysis, the solutions were filter sterilzed and stored at 4°C until use.

7.3. Purification of LTB-Pk by ion-exchange chromatography using a Bio-rad 70 support.

A 25ml Bio-rex® 70 cation-exchange column was prepared by equilibrating the column with 25mM Na_2PO_4, 10mM EDTA, pH 7.0, at room temperature. A 150 ml sample of LTB-Pk (at a concentration of approximately 6mg/l) that had been concentrated by ultrafiltration and dialysed against 25mM Na_2PO_4, 10mM EDTA, pH 7.0, was applied to the prepared column, and when all the sample had been loaded, the column was washed in 10 column volumes of 25mM Na_2PO_4, 10mM EDTA, pH 7.0, to remove any unbound material. Bound protein was eluted by applying a buffer composed of 25mM Na_2PO_4, 10mM EDTA, 0.5M NaCl, pH 7.5. Throughout the purification 5ml fractions were collected, 1µl samples of each fraction spotted onto nitrocellulose and proteinaceous material vizualized as described in

Section 7.2. The fractions containing the highest levels of protein in the column void, were pooled and dialysed against PBS, 10mM EDTA. Likewise, the fractions containing eluted protein were pooled and dialysed. Both samples were filter sterilized and stored at 4°C until use.

8. Expression And Purification Of Recombinant SIV Proteins.

8.1. Plasmids.

The properties of the plasmids pQE917Pk and pQ927Pk are described in detail elsewhere [Hanke *et al.*, 1994]. The recombinant SIV p17 and p27 proteins encoded by these plasmids, respectively, have a short histidine tag at the N-termini and the Pk tag at the C-termini. The expression of the recombinant proteins is under control of the *lac* promotor.

8.2. Expression of His-SIV-Pk proteins in E.coli.

Antibiotic supplemented L-agar plates were prepared as described in Section 1.5. *E.coli* transfected with either plasmid pQE917Pk or pQ927Pk were plated out onto the L-agar plates and incubated at 37°C for 24 hours. Single colonies were selected and used to inoculate a 10 ml volume of supplemented L-broth. The cultures were incubated at 37°C for 18 hours on a rotary shaker, following which, a 1ml sample of the overnight culture was used to inoculate a 1 litre volume of supplemented L-broth. The bacterial cultures were incubated as before until the $O.D._{600}$ reached 0.4-0.6, synthesis of the recombinant SIV proteins were induced by the addition of 0.05mM IPTG (final concentration) to the bacterial cultures and the induction allowed to proceed at 26°C for 2-3 hours (in the case of His-p17-Pk) or 3 hours (in the case of His-p27-Pk). On completion of the induction period, the bacteria were harvested by centrifugation at 12,000g for 25 minutes at 4°C. The resulting supernatant was discarded and the bacterial pellets resuspended in 30ml of ice-cold TN buffer (20mM Tris-HCl pH 8.0, 0.3M NaCl). 10mg/ml of fresh lysozyme was added to the suspension, and following vigorous vortexing, the mixture was incubated on ice for 10 minutes. A 300µl volume of Triton X-100 was added to the lysozyme solution, and the mixture sonicated for two 15 second bursts using a Branson Sonifer B-12 at full power. The bacterial lysates were

centrifuged at 3000g for 15 minutes at 4°C, the supernatant retained and either applied directly to a Nickel $^{2+}$ -nitrilotriacetic acid (Ni^{2+}-NTA) resin or stored at -20°C.

8.3. Purification of recombinant SIV proteins by Ni^{2+}-NTA affinity chromatography.

The protocol for the purification of His-SIV-Pk proteins is an adaptation of the protocol of Smith *et al.*, 1988. A 1 ml sample of Ni^{2+}-NTA resin was transferred into a 50ml universal tube and 30ml of 20mM Tris-HCl, pH 8.5, 0.3M NaCl added. The mixture was centrifuged at 460g for 10 minutes at 4°C in a centrifuge and the supernatant removed. The pelleted resin was resuspended in 30ml of 20mM Tris-HCl, pH 8.5, 0.3M NaCl and the washing step repeated. After the removal of the supernatant, the resin was resuspended in a 30ml volume of the prepared bacterial lysate (Section 8.2) and incubated at 4°C for one hour on an orbital shaker. The mixture was transferred into a 5ml column and the flow through collected. Proteins that failed to bind the nickel resin were then removed by washing the resin in 50 column volumes of 20mM Tris-HCl, pH 8.5, 0.3M NaCl. Purified His-SIV-Pk proteins were eluted by the addition of a 30ml volume of 250mM imidazole, 20mM Tris-HCl, 0.3M NaCl, pH 7.4 to the column. 1 ml fractions were collected throughout the elution step, and the fractions containing the highest level of protein [determined by Naphthlene black staining (Section 7.2)] were pooled and then dialysed overnight against 4 litres of PBS, changing the buffer once. The dialysed proteins were sterilized by filtration through a 0.22μm nylon filter, and stored at 4°C until use.

8.4. Expression of GST-Pk.

Plasmid pGEXcPk codes for the Pk tag attached to the C-terminus of glutathione S-transferase (GST-Pk) [Hanke *et al.*, 1992]. Expression of GST-Pk was achieved using the criteria described for the expression of the SIV recombinant proteins. GST-Pk was harvested from the bacterial lysates of lysozyme treated cells (see above) and stored at -70°C until use.

9. Immunogenicity studies.

9.1. Mice and immunizations.

Balb/c (H-2K^d) mice, were used for all immunogenicity studies.

For parental immunization, mice were aged 6-8 weeks and were injected intraperitoneally with the vaccine formulations in a final volume of 200µl PBS.

For vaccines that were administered in association with alum, the alum precipitate was formed and the antigens allowed to absorb. Typically, 900µl of PBS was mixed with 30µl of 1M $NaHCO_3$ and 60µl of 10% (w/v) $Al_2(SO_4).K_2SO_4.2H_2O$ added to the solution dropwise, gently agitating the mixture during the application of the $Al_2(SO_4).K_2SO_4.2H_2O$. The precipitate was then centrifuged at 2,500g for 5 minutes, and then resuspended in 900µl of PBS containing the appropriate concentration of antigen. The antigen was allowed to adsorb to the alum for one hour at 4°C, then the solution was used directly for immunisation, using 200µl per mouse.

Balb/c mice aged 10-12 weeks were used for intranasal vaccination. All vaccine formulations were prepared in a final volume of 50µl PBS (unless otherwise stated). The mice were gently anaesthetised by exposure to diethyl ether, and the vaccines slowly administered, dropwise, using a micropipette, to the outer mandrils of the nasal cavity.

9.2. Measurement of immune sera by the enhanced chemilumescence (ECL)-based immune assay.

This assay is an adaptation of the protocol originally described by Randall and colleagues [1994]. 7 x 5 cm^2 sheets of nitrocellulose (Gelman Sciences) were placed in petri dishes and a 10 ml solution of carbonate buffer pH 9.6 (1.59g/l Na_2CO_3, 2.93g/l $NaHCO_3$) containing the purified target antigens at the concentrations shown below. Following a two hour incubation at 37°C, on a orbital shaker, the carbonate buffer was removed and the filters washed extensively in PBS. The remaining protein binding sites on the nitrocellulose sheets were blocked by incubating the sheets in a 20 ml volume of blocking solution (5% semi-skimmed milk, 20mM Tris-HCl, pH 7.6, 140mM NaCl, 0.1% Tween-20) for 15 minutes at room temperature on an orbital shaker. The filters were washed in PBS, then blocked for a

further 10 minutes at room temperature in blocking buffer. The excess blocking solution was removed and the filters washed in PBS, as before. The test sera was serially diluted in blocking buffer (see Results for dilutions) and added to the wells of terasaki plates (Sterilin) in 12µl volumes. The terasaki plates were overlaid with the antigen-coated sheets and a 3MM Whatman filter, cut to the same size as the nitrocellulose sheets, was moistened in PBS and then placed on top of the nitrocellulose. A second terasaki plate was carefully placed over the sandwich, making sure the wells of both plates were in alignment. The plates were tightly clipped together and then inverted to bring the sera into contact with the antigen-coated nitrocellulose sheets. Following one hour incubations at room temperature on an orbital shaker, the nitrocellulose sheets were removed from the plates and placed in a petri dish containing 20ml of blocking buffer. The sheets were then washed twice in blocking buffer for 15 and 10 minute intervals, followed by a single wash in PBS, before being incubated with a 10 ml volume of blocking buffer containing a 1:2000 dilution of an horseradish peroxidase (HRP)-conjugate (either anti-mouse Ig or Protein A), for 30 minutes at room temperature on an orbital shaker. On completion of the incubation, the nitrocellulose sheets were washed in blocking buffer and PBS as before. The nitrocellulose sheets were transferred into a fresh petri dish and incubated for two minutes at room temperture in a 5 ml volume of enhanced chemiluminscence (ECL) reagents (Amersham) that had been mixed according to the manufacturer's recommendations. The nitrocellulose sheets were briefly dried, placed in a plastic bag and then exposed to X-ray film (Fuji) adjacent to an intensifying screen. After various incubation times, the X-ray film was developed in an automatic developer (Kodak).

Target Antigen	Concentration
His-p27-Pk	4µg/ml
His-p17-Pk	4µg/ml
GST-Pk	20µl of cell lysate
ovalbumin	1mg/ml
*LTB-Pk	5µg/ml

* For sheets coated with LTB-Pk, all nitrocellulose filters were first of all incubated in 10ml of carbonate buffer containing 1.5µg/ml GM1-monosialoganglioside for one hour at 37°C, prior to the addition of LTB-Pk. This helped to potentiate the binding of LTB-Pk to the nitrocellulose.

9.3. Measurement of the secretory immune response by ECL-immune assays.

Mice were injected intraperitoneally with 2% (v/v) pilocarpine, diluted 1:3 in PBS, in a final volume of 200µl PBS, to stimulate salival flow. Approximately 100µl of saliva was collected in a 1.5ml vial, and following high-speed centrifugation in a bench-top centrifuge, stored at -20°C until required.

Assessment of antigen-specific secretory antibody was performed using an adaption of the ECL-based immune assay above. Briefly, the saliva was serially diluted in blocking buffer and incubated with antigen-coated nitrocellulose sheets. Antigen-specific secretory antibodies were detected by incubating the nitrocellulose sheets with goat anti-mouse IgA, followed by an HRP-conjugated donkey anti-goat antibody and autoradiography.

9.4. Lymphocyte proliferation assays.

9.4.1. Preparation of splenocytes.

The spleens were placed on a sterile nylon mesh in a petri dish (Sterilin) containing 10ml of PBS. The splenocytes were liberated by mashing the tissue through the nylon mesh using a flat-bottomed plunger from a 10ml sterile syringe (Sterilin) and the cells placed in 30ml universal tubes. The universal tubes were allowed to stand for 3 minutes to allow cell debris and fatty tissue to pellet, then the supernatant was gently transferred to fresh 30ml universal tubes using a pasture pipette. The cells were centrifuged at 400g for 10 mins at 20°C, and then resuspended in 10ml of 0.85% ammonium chloride solution to lyse the red blood cells. After a 5 minute incubation at room temperature, the ammonium chloride/cell suspension was under-layed with 10ml of foetal calf serum (FCS) and centrifuged at 400g, 20°C for 10 minutes. The supernatant was discarded and the cells were resuspended in 20ml of PBS and centrifuged as before. This last step was repeated four times. The splenocytes

were then resuspended in 10ml of PBS and the suspension vigorously pipetted, to break up any clumps of cells. A 20µl sample of the cell suspension was mixed with 20µl of Trypan blue (0.2% Trypan blue: 4.25% NaCl, 4:1), the mixture placed on a haemocytometer and live cells (cells that did not stain blue) enumerated. The cells were diluted in T cell medium to a final concentration 10^6 cell/ml and used directly in stimulatory assays.

9.4.2. Lymphocyte stimulation and measurement of DNA synthesis.

Purified His-p17-Pk or His-p27-Pk were diluted to a concentration of 4µg/ml in T-cell medium [RPMI-1640 (Gibco) supplemented with 2mM L-glutamine, 100 U penicillin, 100µg streptomycin, 5 x 10^{-5}M β-mercpatoethanol] and 200µl added to the top row of an appropriately labelled round-bottomed, 96-well microtitre plate, then serial diluted 1:2 in T cell medium to give a final volume of 100µl in each well. Purified LTB-Pk was diluted in T cell medium to a concentration of 1µg/ml, then diluted in the wells of a round bottomed 96-well microtitre plate as described for the His-SIV-Pk antigens.

100µl of the cell suspension prepared in Section 9.4.1, was added, in triplicate, to each well of the antigen containing microtitre plates. The plates were incubated for 5 days in a humidified incubator at 37°C, 5% CO_2 and during the last 18 hours of the stimulation, 0.5µCi [^{3}H]-thymidine was added to each well. The cells were harvested onto glass microfibre filters using an Ilacon cell harvester and the amount of [^{3}H]-thymidine incorporated into the DNA of the proliferating cells was determined by scintillation counting.

9.5. Cytokine-ELISA Assays.

9.5.1. Preparation of culture supernatants.

10^7 cells, prepared from murine spleens were resuspended in 500µl of T cell medium (Section 9.4.1) and added to the wells of 24-well microtitre plates (Sterilin). The cells were stimulated with the antigens (see Results) for 5 days at 37°C/5%CO_2 in a humidified incubator. 100µl samples of the culture supernatant were removed on day five of the stimulation and stored at -20°C until assayed for cytokines.

9.5.2. Detection of IL-2, IL-5 and IFN-γ.

Cytokine production was quantified using sandwich ELISA techniques. Enhanced protein binding 96-well ELISA plates (Dynalab Immulon 4) were coated overnight at 4°C with 50μl of monoclonal anti-cytokine antibody (Pharmingen) at pre-determined optimal concentrations (see below) in 0.1M carbonate buffer, pH 9.6. The plates were then washed twice with PBS containing 0.05% Tween 20 (PBS/Tween), after which non-specific protein binding sites were blocked by incubation with 200μl of PBS containing 10% FCS for 1 hour at 37°C. Following blocking, the plates were washed three times in PBS/Tween and samples and standards (recombinant murine cytokines), diluted in T-cell medium, were added to individual wells in a volume of 50μl and incubated at 37°C for 3 hours. The plates were washed 4 times in PBS/Tween and 50μl/well of biotinylated anti-murine cytokine antibody, diluted in PBS/10% FCS was added at the concentrations shown in Table. After incubation at room temperature for one hour, the plates were washed four times in PBS/Tween and 100μl/well extravidin-peroxidase in PBS/10% FCS was added to each well at 2μg/ml. Following a final incubation for one hour at room temperature, the plates were washed six times in PBS/Tween, before 100μl of TMB peroxidase substrate was added to each well. The adsorbance was measured at 630nm using an Dynatech MR5000 automatic microplate reader and cytokine concentrations in test supernatants were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines.

Cytokine specific antibody	Capture concentration	Detection concentration
anti-IL-2	2μg/ml	1μg/ml
anti-IL-5	4μg/ml	4μg/ml
anti-IFN-γ	2μg/ml	1μg/ml

9.6. FACscan analysis.

Mixed lymphocyte populations, prior to, or after a five day stimulation with the appropriate antigen (see Results) were placed in 30ml universal tubes and centrifuged at 400g for 10 minutes at 20°C. The pelleted cells were resuspended in 10ml of PBS and the number

of cells present enumerated as described in Section 9.4.1. Aliquots of 10^7 cells were removed and placed in 1.5ml eppendorf vials, centrifuged at high speed in a microfuge and then resuspended in 100µl of PBS containing a 1:100 dilution of the appropriate FITC-labelled antibody (anti-mouse CD4, anti-mouse CD8 or anti-mouse Ig; see Results). The suspensions were incubated on ice for one hour, then centrifuged at 400g for 5 minutes in a microfuge. The pelleted cells were resuspended in 500µl of PBS and centrifuged as before. This washing step was repeated three times to ensure no uncomplexed FITC-labelled antibody remained. After the final wash, the cells were resuspended in 500µl of PBS and transfered into Becton Dickson FACSscan tubes (Falcon 2500). 10,000 events were collected using the Becton Dickson Consort 30 program and the percentage of fluorescent cells in 10,000 events (collected using the Consort 30 program) was measured using the LYSYS program.

9.7. Enzyme-linked Immunospot (ELISPOT) assay for the detection of antibody secreting cells.

The ELISPOT assay was developed for the detection of antibody secreting cells is an adaptation of the protocol described by Czerkinsky *et al.*, 1983.

9.7.1. Preparation of lung cells

Mice were killed by anaesthesia and their lungs removed under aseptic conditions and placed in a 30ml universal (Sterilin) containing 10ml of sterile PBS. All other procedures were performed in a sterile cabinet. The lungs were placed into a 25mm^2 petri dish containing 10ml of PBS and the lungs were gently washed to remove excess blood. The tissue was then removed to another 25mm^2 petri dish containing 10ml PBS and cut into small 2mm sections using a scalpel and forceps. The outer epithelial layer of the lungs were removed by placing the dissected lung pieces into a 10ml universal tube (Sterilin) and adding a solution of PBS containing 10mM $MgCl_2$, 0.5U/ml collagenase A (Boehringer Mannhein) and 0.025% DNaseI (Boehringer Mannhein) (1ml of solution per lung). After a 45 minute incubation at 37°C under gentle agitation, the digested tissue was then placed on a nylon mesh in a 25mm^2 petri dish containing 10ml of PBS and the cells released by mashing the tissue through the mesh using a plunger from a 10ml syringe. The cells were placed into 30ml universal tubes,

the cell debris pelleted by standing the universal tubes upright for 3 minutes and the supernatant was gently removed to fresh 10ml universal tubes. The cells present in the supernatant were centrifuged at 20°C for 10 mins at 470g and then resuspended in red cell lysis buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.1mM EDTA, pH 7.4), using 2ml of buffer per lung. After 10 minute incubations at room temperature, the cells were centrifuged at 470g for 10 mins and the supernatant discarded. The cells were resuspended in 10ml of PBS and centrifuged as before. This step was repeated twice more. The cells were then resuspended in 1ml of PBS per lung and the number of live cells enumerated following trypan blue staining (Section 9.4.1).

9.7.2.Preparation of spleen cells.

Spleen cells were prepared as described in Section 9.4.1.

9.7.3 Detection of antibody-specific spot forming cells (S.F.C.)

Purified His-p27-Pk was diluted to a concentration of 4µg/ml in carbonate buffer and added to the wells of a 96-well, flat-bottomed microtitre plate in 100µl volumes.After a 2 hour incubation at 37°C, the plates were washed three times in PBS. Excess liquid was removed by shaking the plates and the remaining binding sites blocked by adding 200µl volumes of 1% BSA in PBS to each well. Following a three hour incubation at 37°C, the blocking solution was decanted and the plates were washed three times in PBS.

The cells were diluted to an appropriate density (10^4 to 10^6 cells/ml) in Iscove's medium (Gibco), supplemented with 100U/ml penicillin, 100µg/ml streptomycin and added to the plates in 100µl volumes. The plates were incubated overnight at 37°C, 5% CO_2 in a humidified incubator, following which the cells were discarded and any remaining bound cells lysed by incubation in PBS containing 0.5% Tween-20. A 1:1000 dilution of anti-mouse IgM, IgG or IgA antibody [diluted in PBS containing 0.1% BSA (PBS/BSA)] was added to each well in 100µl volumes and the plates were incubated at room temperature for 3 hours. The plates were washed three times in PBS containing 0.05% Tween-20, then a 1:1000 dilution (in PBS/BSA) of an alkaline-phosphatase conjugated antibody was added to each well in

100µl volumes. The plates were incubated at 37°C for three hours, and after washing the plates three times in PBS/Tween followed by twice in distilled water, antibody-antigen complexes were visualized by the addition of 100µl of alkaline phosphatase-substrate [100mg BCIP (5-bromo-4-chloro-3-indoyl phosphate) dissolved in 100ml AMP buffer (9.58ml 2-amino-2-methyl-1-propanol {Sigma}, 15mg $MgCl_2$, 0.1mg sodium azide, made up to 100ml in distilled water)]. The plates were incubated at 37°C until blue spots became visible, and when the spots were fully developed, the plates were gently washed in distilled water and the number of spot forming cells enumerated by examining the plates under a dissection microscope.

RESULTS

SECTION A. Expression and Characterisation of LTB-Pk.

A.1. Introduction

Section A of the results describes the addition of a 14 amino acid tag, termed Pk, to the carboxy terminus of the B subunit of the heat-labile enterotoxin (LTB) from *Escherichia coli* (*E.coli*) and the expression of the novel fusion protein, LTB-Pk, in both *E.coli* and the non-pathogenic marine *Vibrio*, *Vibrio sp.60*. The influence that the Pk epitope exerts on the physical properties attributed to authentic LTB are investigated.

A.2. Construction of expression vector pTRH-Pk

The plasmid pTRH101R is a controlled expression vector, into which the coding sequence for the LTB gene, containing a short oligonucleotide sequence addition at its 3'-end, was cloned downstream from the isopropyl-β,D-thiogalactopyranoside (IPTG)-inducible *tac* promoter. This extended sequence contained five unique endonuclease restriction sites for cloning additional sequences onto the 3'-end of the gene. Cleavage of pTRH101R at the HindIII and SpeI restriction sites facilitated the insertion of a double-stranded oligonucleotide sequence encoding a 14-amino acid tag, termed Pk. (The amino acid sequence of the Pk tag is shown in Fig. 8) The resulting novel plasmid pTRH-Pk, encoding recombinant LTB-Pk, was transfected into either *E.coli* or *Vibrio sp.*60 (this latter transfection was kindly performed by Dr. T.R. Hirst, University of Kent, Canterbury).

A.3. Comparison of the expression and cellular localisation of LTB-Pk in E.coli and Vibrio sp.60

E.coli and *Vibrio sp.* 60 have both been shown to be capable of expressing native LTB molecules, the latter host having the additional advantage that the LTB molecules are secreted into the external milieu [Leece *et al.*, 1988]. To determine whether the

recombinant protein LTB-Pk could be similarly expressed, *E.coli* and *Vibrio sp.*60 harbouring plasmid pTRH-Pk, were cultured in ampicillin supplemented L-broth at 37°C and 30°C (respectively) and when the $O.D_{600}$ reached 0.4, LTB-Pk was induced by the addition of 0.5mM IPTG (final concentration) to the bacterial cultures.

After a 4 hour induction, 1ml samples from each culture were removed, the bacteria separated from the culture media by centrifugation and the culture media retained. The bacterial pellets were then resuspended in 1ml of 20mM Tris, 0.3M NaCl, pH 8.0 and the intracellular proteins released by lysozyme treatment. As a control, bacteria containing plasmid pTRH-Pk, but not exposed to IPTG, were similarly treated. Equivalent volumes from the protein containing medium and cell lysate for each culture, were electrophoresed through a 15% (w/v) SDS-polyacrylamide gel and the resulting polypeptide chains electroblotted onto a nitrocellulose filter. The presence of LTB-Pk was visualised by probing the blots with the anti-Pk monoclonal antibody (MAb), MAb SV5-P-k and detecting bound antibody by enhanced chemiluminiscence (ECL) Western blotting assay, utilising horseradish peroxidase (HRP)-conjugated Protein A and autoradiography (Fig.9).

A signal was present on the autoradiograph, representing the interaction of MAb SV5-P-k with the Pk epitope of the recombinant protein in both *E.coli* and *Vibrio sp.* 60 after exposure to IPTG, demonstrating that LTB-Pk can be expressed in both organisms. However, following a four hour induction with IPTG, the cellular location of LTB-Pk differed for the respective bacteria. In the case of *E.coli* , LTB-Pk was detected in the cellular lysate (lane 3) but not in the culture medium (lane 2), suggesting that LTB-Pk remained cell associated in this organism. In contrast to *E.coli*, only a small proportion of LTB-Pk was present in the cell lysate from *Vibrio sp.* 60 cultures (lane 6), as shown by the weak band on the autoradiograph, the vast majority of the fusion protein being secreted into the culture medium (lane 5).

When the cell lysate from *E.coli* control cultures (lane 1) and the culture medium from *Vibrio sp.* 60 control cultures (lane 4) were similarly analysed, no LTB-Pk was detectable, demonstrating that LTB-Pk expression is under control of the *tac* promoter.

	leu	gly	lys	pro	ile	pro	asn	pro	leu
AG	CTG	GGA	AAG	CCG	ATC	CCA	AAC	CCT	TTG
	C	CCT	TTC	GGC	TAG	GGT	TTG	GGA	AAC

leu	gly	leu	asp	ser	thr	AMB	
CTG	GGA	TTG	GAC	TTC	ACC	TAG	A
GAG	CCT	AAC	CTG	AAG	TGG	ATC	TGA TC

Figure 8. DNA linker coding for the Pk tag

The figure shows the sequences of the positive and negative strand oligonucleotides, which were designed to give after annealing HindIII-compatible and SpeI cohesive ends. The Pk tag linker codes for amino acids 95-108 derived from SV5 P and V proteins (shown in bold letters). Underlined region corresponds to the sequences recognized by MAb SV5-P-k.

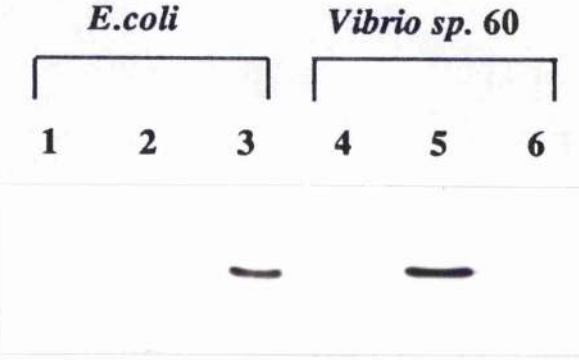


Figure 9 . Expression and cellular localization of LTB-Pk.

Plasmid pTRH-Pk was transferred into *E.coli* and *Vibrio sp.* 60, and the bacteria were cultured in the absence (lanes 1&4) or presence (lanes 2-3& 5-6) of 0.5mM IPTG for 4 hours. The bacteria were then harvested by centrifugation and the intracellular proteins released by lysozyme treatment. The expression of LTB-Pk in the culture medium (lanes 2&5) and the cellular lysate (lanes 3&6) for the respective bacteria, was detected with MAb SV5-P-k in an ECL-Western blotting assay utilizing HRP-conjugated Protein A and autoradiography.

A.4. Oligomerisation of altered B subunits.

At room temperature, native LTB exists as stable pentamers in the ionic detergent sodium dodecyl sulphate (SDS), but dissociates into its constitutive monomers upon boiling [Hardy *et al.*, 1988]. To determine whether LTB-Pk also existed as a heat-labile oligomer, the mobility of heat-treated and untreated LTB-Pk was investigated.

Equal samples of culture media from IPTG induced *Vibrio sp.* 60 cultures, were mixed with an appropriate volume of 4X disruption solution and then either heated at 100°C for five minutes or left at room temperature. The prepared samples were added to duplicate gels, one of which was stained with Coomassie blue [Fig.10 (a)], the other, Western blotted onto nitrocellulose and the presence of LTB-Pk detected with the anti-Pk MAb in an ECL-Western blotting assay [Fig.10 (b)].

Two major protein species were detectable by Coomassie blue stain in the sample from *Vibrio sp.*60 culture medium that had been boiled prior to the addition to the gel [Fig.10(a), lane 2]. Of the two proteins seen, the protein with faster mobility, migrated with an apparent molecular weight of approximately 14kD (lane 1) and was recognised by MAb SV5-P-k in the corresponding autoradiograph [Fig.10(b), lane 1]. However, in samples that had not been exposed to heat treatment prior to SDS-PAGE, a single protein migrated with an apparent molecular weight of approximately 45kD, was detectable in both the Coomassie stained-gel [Fig.10 (a), lane 2] and the Western blot [Fig.10(b), lane 2,]. Similar Western blot analysis of LTB-Pk expressed in *E.coli*, presented identical results to that of *Vibrio sp.*60 expressed LTB-Pk (data not shown).

These findings clearly show that LTB-Pk is a heat-labile oligomer, thereby demonstrating that the presence of the Pk epitope at the C-terminus of LTB does not prevent subunit-subunit association of the LTB-Pk monomers. In addition, Coomassie staining of the proteins detectable in the culture supernatant of IPTG-induced *Vibrio sp.*60, suggests that LTB-Pk and a second protein of unknown function, predominate.

A.5. Binding of LTB-Pk to the glycolipid, GM1-ganglioside.

Native LTB has been shown to bind to gangliosides and certain sugars, presumably through the affinity of the enterotoxin subunit for galactose residues [Finkelstein and Clements, 1979]. It is this property that is thought to target LTB onto cells bearing these molecules, promoting the uptake and potentially the immunogenicity, of LTB-fused epitopes. To further characterise LTB-Pk, the ganglioside-binding properties of the recombinant protein were analysed in a GM1-ganglioside-based ELISA (GM1-ELISA) assay.

A 96-well microtitre plate was coated with either GM1-ganglioside or a control antigen, ovalbumin, and then incubated with LTB-Pk-containing medium. The binding of the fusion protein to either antigen was detected by incubation with MAb SV5-P-k followed by HRP-conjugated Protein A and HRP substrate and measuring the adsorbance at 450nm. As secondary controls, the antigens were incubated MAb SV5-P-k or PBS alone, prior to the addition of HRP-conjugated Protein A, to ascertain the degree of non-specific binding of the MAb and HRP-conjugate, respectively, to either the GM1 ganglioside or ovalbumin.

As shown in Fig. 11, only low levels of adsorbance were detectable at 450nm in the control wells of GM1 alone (lane 1) or GM1 incubated with MAb SV5-P-k (lane 2) demonstrating that neither the HRP-conjugated Protein A nor the anti-tag MAb alone, were capable of binding to GM1 ganglioside. However, a prior incubation between GM1 and LTB-Pk-containing medium, before the addition of the antibody and Protein A, resulted in a sharp increase in adsorbance (lane 3) demonstrating that LTB-Pk bound to the glycolipid. Confirmation of the specificity of the interaction between the glycolipid and LTB-Pk was provided when LTB-Pk was assayed against a control antigen, ovalbumin (lane 6), where the level of adsorbance was below background levels (lanes 4 & 5).

Thus, the addition of the Pk-epitope to LTB does not prevent interaction of the enterotoxin subunit with its glycolipid substrate.

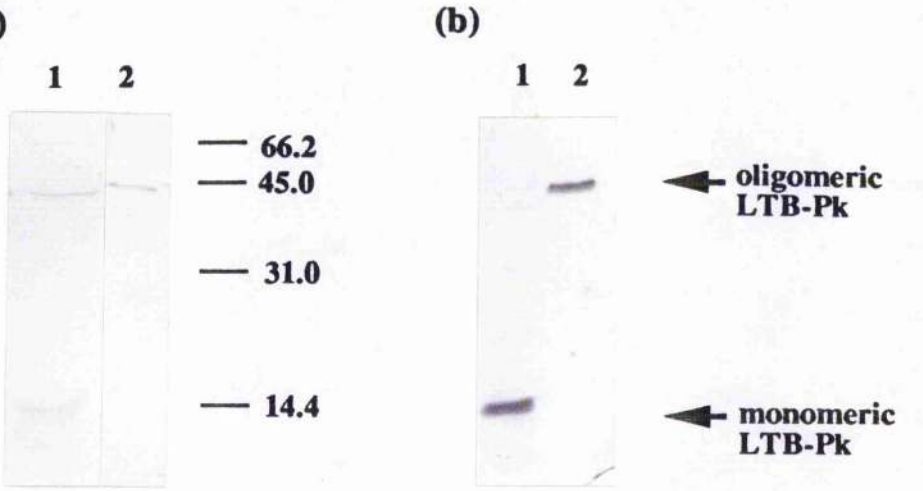


Figure 10. Oligomerization of LTB-Pk.
A sample of the culture medium from *Vibrio sp.*60 induced cultures containing plasmid pTRH-Pk was mixed with an appropriate volume of 4X disruption buffer, then either boiled for 5 mins (lanes 1) or left at room temperature (lanes 2) prior to 15% (w/v) SDS-PAGE. The presence of LTB-Pk was visualized by Coomassie blue staining (Panel (a)) or Western blotting (Panel (b)). Molecular marker weights are shown on the right-hand-side of Panel (a).

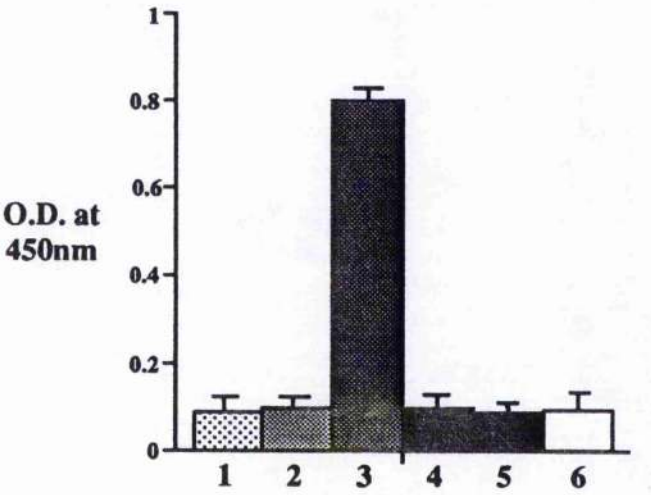


Figure 11. Affinity of LTB-Pk for GM1-ganglioside.
96-well microtitre plates were coated with either 1.5µg/ml GM1 ganglioside (lanes 1-3) or 1mg/ml ovalbumin (lanes 4-6) then incubated with PBS alone (lanes 1&4), MAb SV5-Pk (lanes 2&5) or LTB-Pk-containing culture medium (lanes 3&6). On completion of the incubation, MAb SV5-P-k was added to all wells, followed by an HRP-conjugated Protein A antibody and the adsorbance at 450nm was measured after the addition of substrate. The results are presented as the mean ± standard deviation for duplicate cultures.

A.6. Optimising the expression of LTB-Pk.

One of the considerations in developing an expression system is to establish the ideal conditions that lead to optimum production of the desired protein, this being particularly important if the protein is to be used in substantial amounts. To determine the ideal conditions for the expression of LTB-Pk in *E.coli* and *Vibrio sp.*60, the influence of the (a) temperature and (b) IPTG concentration on the synthesis of LTB-Pk in the respective bacteria, was investigated.

Identical 200ml cultures of *E.coli* and *Vibrio sp.* 60, were cultured until the $O.D._{600}$ reached approximately 0.4 and then the fusion protein was induced for 4 hours at either 37°C or 30°C, with a final concentration of IPTG ranging from 1mM-0.05mM. On completion of the induction period, a 1ml sample was removed from each culture and the level of LTB-Pk expression was determined by Western blotting.

Comparison of the signal intensities on the autoradiograph after probing the blots with the anti-Pk MAb, suggested that a reduction in temperature favoured increased yields of LTB-Pk for both bacterial species, the overall expression of LTB-Pk seeminged higher in *Vibrio sp.* 60 than in *E.coli* (Fig. 12). Interestingly, two bands were detectable on the autoradiograph in *E.coli* cultures induced at 30°C, the band with a slightly higher molecular weight possibly representing precursor molecules of LTB-Pk, where the N-terminal signal sequence, which targets precursor LTB molecules to the cytoplasmic membrane prior to secretion [Palva *et al.*, 1981], was not efficiently cleaved from LTB-Pk. In contrast to *E.coli* , in *Vibrio sp.* 60, LTB-Pk was consistently detectable as a single band on autoradiography at either 30°C or 37°C.

The influence of the IPTG concentration on the synthesis of LTB-Pk in the bacteria was particularly noticeable at 30°C. In *Vibrio sp.* 60, there is an apparently uniform level of LTB-Pk productivity throughout the IPTG range added to the cultures, but, in *E.coli*, maximum yields of LTB-Pk were obtained only at higher IPTG concentrations of 1-0.5mM. However, this was not the case when LTB-Pk was induced at 37°C in *E.coli* where there seemed to be little difference in the level of LTB-Pk synthesis following

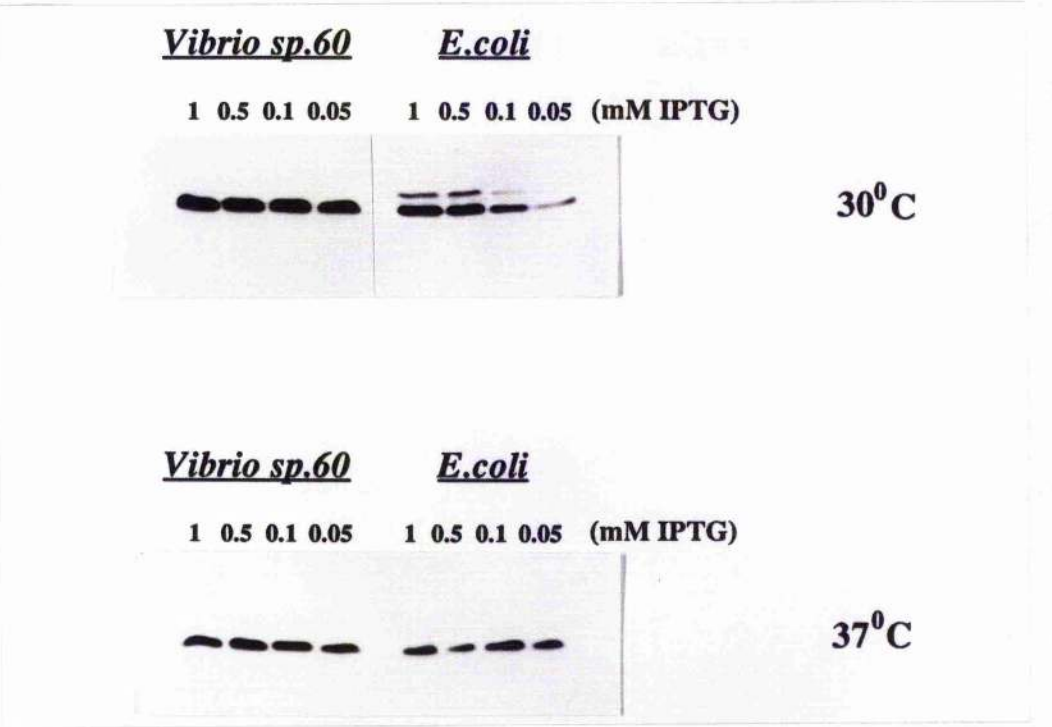


Figure 12. Effect of IPTG concentration and temperature on the expression of LTB-Pk in *Vibrio sp.*60 and *E.coli*. Cultures of*Vibrio sp.*60 or *E.coli* harbouring the plasmid pTRH-Pk were induced for 4 hours at either 30°C or 37°C, with a final concentration of IPTG ranging from 0.05mM-1.0mM. On completion of the incubation period, the bacteria were harvested by centrifugation and 15µl samples were removed. The samples were boiled for 5 minutes, then electrophoresed through a 15% (w/v) SDS-polyacrylamide gel. The level of expression of LTB-Pk in the culture medium (for *Vibrio sp.*60) or the cell lysate (for *E.coli*) was determined by Western blotting, using MAb SV5-P-k as the detection antibody.

addition of 1mM IPTG or 0.05mM IPTG. Similarly, differing IPTG concentrations had little effect on the overall expression of LTB-Pk in *Vibrio sp.*60 at 37°C. These results suggest that *Vibrio sp.*60 is a more suitable expression vector than *E.coli* for the expression of LTB-Pk.

On the basis of these findings, the expression of LTB-Pk in *Vibrio sp.* 60 was further analysed to determine at what time after induction maximum accumulation of the fusion protein occurred in the culture medium. A 100ml culture of *Vibrio sp.*60 was grown until the $O.D._{600}$ reached 0.4, then the fusion protein was induced at 30°C by the addition of 0.05mM IPTG to the bacterial culture. At the times indicated in Fig.13, a 1ml sample of the culture was removed, the bacteria pelleted by centrifugation, and the culture medium retained. 15µl samples of the culture medium were electrophoresed through a SDS-polyacrylamide gel, the polypeptides transferred onto nitrocellulose and the level of LTB-Pk expression in relation to the induction time was determined by Western blotting. (Fig.13).

It was evident from the resulting autoradiograph, that LTB-Pk was detectable in the culture medium as rapidly as 30mins after the addition of IPTG to the culture, and the amount of LTB-Pk accumulating in the culture medium rose rapidly over the next two hours. Thereafter, although the slight increasing intensity in the autoradiograph signals implied that LTB-Pk was still being synthesised and secreted into the culture medium over an 18 hour induction period, the actual rate of LTB-Pk synthesis was more constrained. Significantly, LTB-Pk was detectable at all times with MAb SV5-P-k as a single protein band on the autoradiograph, suggesting that the fusion protein was not degraded, even after a prolonged 18 hour induction.

The amount of LTB-Pk present in the culture supernatant at various times after induction with IPTG, was quantitated in a GM1-ELISA assay, using purified LTB-Pk as the standard in the assay.

As shown in Table 1, the concentration of LTB-Pk detectable in the culture medium increased in correlation with the length of the induction period, the maximum

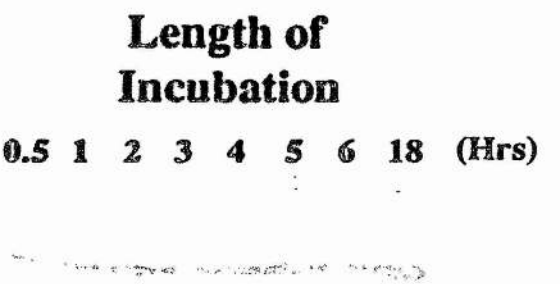


Figure 13. Influence of induction times on the expression of LTB-Pk
*Vibrio sp.*60 harbouring plasmid pTRH-Pk was induced with 0.05mM IPTG at 30^0 C. At the times indicated above, a 1ml sample was removed, the bacteria pelleted by centrigugation and the supernatant retained. Equal volumes from each culture was subjected to SDS-PAGE through a 15% SDS-polyacrylamide gel, the polypeptides electroblotted onto nitrocellulose paper and the blot probed with MAb SV5-P-k. Bound antibody was detected with HRP-conjugated Protein A, ECL and autoradiography.

Time after Induction	Concentration of LTB-Pk
2 hours	1.1 mg/l
3 hours	1.3 mg/l
4 hours	3.1 mg/l
5 hours	4.9 mg/l
6 hours	8 mg/l
18 hours	12 mg/l

Table 1. Concentration of LTB-Pk in relation to time.
The concentration of LTB-Pk secreted from induced *Vibrio sp.* 60 was quantitated by GM1-ELISA assays.
Doubling dilutions (starting at a concentration of 1:10) of the LTB-Pk containing culture supernatant was performed along GM1-coated wells, and bound protein was detected with MAb SV5-P-k, followed by HRP-conjugated Protein A. Purified LTB-Pk (3mg/ml) was employed as the standard in the quantitation.

level of recombinant protein obtained following an 18 hour induction with IPTG was approximately 12mg/litre.

A.7. Stability of LTB-Pk following expression in Vibrio sp.60.

The Western blot and GM1-ELISA assays (Section A.6) provided evidence that LTB-Pk can be expressed in *Vibrio sp.*60 to high levels. However, these assays relied on the interaction of the Pk determinant of LTB-Pk with the anti-Pk MAb SV5-P-k and thus, are insufficient at determining whether the culture medium contained a mixture of LTB-Pk and LTB (degraded LTB-Pk) molecules.

To investigate the stability LTB-Pk following expression in *Vibrio sp.*60, immune precipitation reactions were performed. *Staphylococcus aureus (St.aureus)* was saturated with MAb SV5-P-k, then 21µl volumes of the culture supernatant obtained after the 6 and 18 hour inductions were mixed with the *St. aureus*-MAb complex. The proteins that precipitated onto the bacteria in the presence of MAb SV5-P-k and the proteins present in the unprecipitated supernatant were analysed by Coomassie blue staining, following SDS-PAGE (Fig.14). As a control, *St. aureus* was mixed with a 21µl sample of the culture supernatant obtained after an 18 hour induction with IPTG, to establish whether LTB-Pk was capable of binding to the bacteria in the absence of MAb SV5-P-k.

Analysis of the precipitate from the reaction between *St. aureus* and LTB-Pk, revealed that the fusion protein was unable to bind to the bacteria in the absence of MAb SV5-P-k (lane 5) and therefore, LTB-Pk remained in the non-precipitated fraction (lane 6). However when LTB-Pk, present in the samples removed after 6 and 18 hour inductions, was incubated with MAb SV5-P-k-saturated *St. aureus*, LTB-Pk was detectable in the precipitate (lanes 3 & 1, respectively). Further, examination of the non-precipitated fraction yielded no evidence of degraded LTB-Pk products (lanes 4 & 2, respectively).

Hence, in terms of protein stability, these results provide evidence that LTB-Pk can be expressed in *Vibrio sp.*60 for prolonged periods (up to 18 hours), without any obvious concomitant degradation.

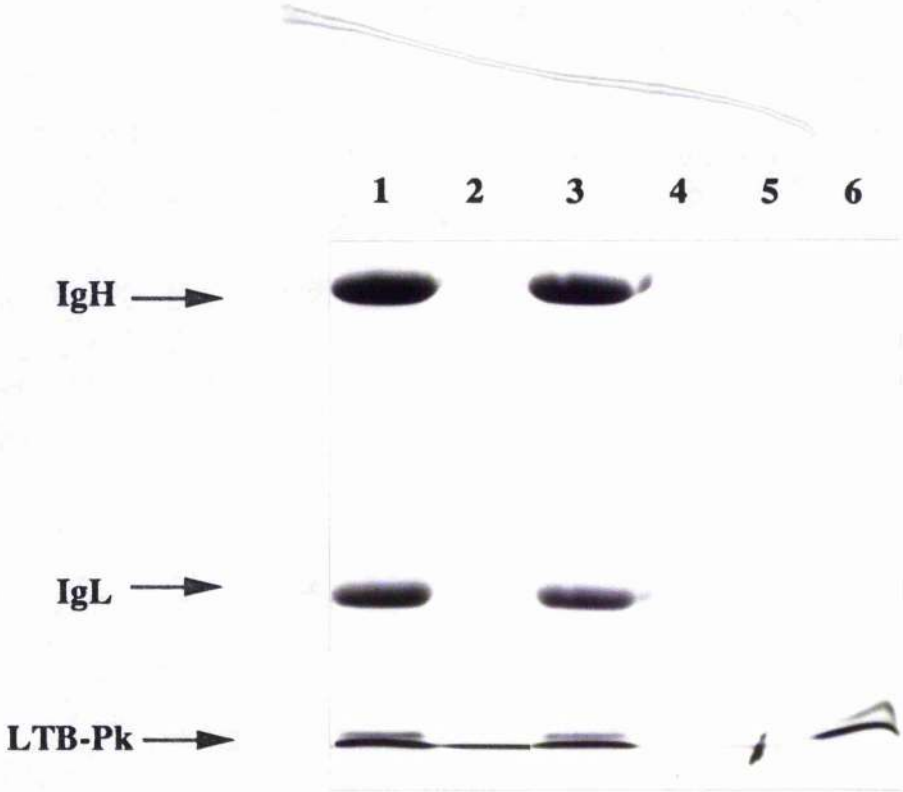


Figure 14. Stability of LTB-Pk following expression in *Vibrio sp.*60.

*Vibrio sp.*60 harbouring plasmid pTRH-Pk was cultured at 30°C and synthesis of LTB-Pk induced by the addition of 0.05mM IPTG to the bacterial culture. 21µl samples of the culture medium were removed after 6 (lanes 3 &4) and 18 (lanes 1,2 & 5,6) hour inductions, and mixed with MAb SV5-P-k-saturated *St aureus* (lanes 1 & 3)or *St. aureus* alone (lane 5). Both the immune-precipitated complexes and non-precipitated supernatants were electrophorised through a 15% (w/v) SDS-polyacrylamide gel, and the protein bands stained with Coomassie blue.

SECTION B. Concentration and Purification of LTB-Pk.

B.1. Introduction

In Part A of the results, it was demonstrated that a 14 amino acid tag, termed Pk, could be attached to the C-terminus of LTB, and the novel LTB-Pk fusion protein could be subsequently expressed and secreted in *Vibrio sp.* 60. In addition, LTB-Pk displayed physiological properties akin to native LTB molecules. In Part B, the results concentrate on the purification strategies that have been employed to generate either a partially or highly purified sample of LTB-Pk and the stability of the fusion protein LTB-Pk after such manipulations.

B.2. Concentration of LTB-Pk.

Although the secretion of LTB-Pk from *Vibrio sp.*60 bypasses the need for cellular lysis as an initial step in purification procedures, it does present the problem that LTB-Pk has to be purified from volumes of culture media greater than 1 litre, depending on the amount of protein desired. Such large volumes are difficult to work with, and highlight the need for a procedure that will concentrate LTB-Pk into a volume that is easier to handle in subsequent purification protocols. One of the oldest protein concentration methods known for recovering and purifying proteins, is precipitation by neutral salts e.g. ammonium sulphate [see '*Protein Purification Applications*' 1990], and the early concentration protocol, described in the next section, was based on the precipitation of LTB-Pk from *Vibrio sp.* 60 culture medium using ammonium sulphate at 50% saturation.

However it soon became apparent that, although ammonium sulphate [$(NH_4)_2SO_4$] precipitation was obviously quite successful in concentrating LTB-Pk from culture supernatants, to employ such a technique for large scale preparations of 1 litre or more, would be both extremely time-consuming and involve vast quantities of $(NH_4)_2SO_4$. It had been demonstrated in our laboratory that monoclonal antibodies could be concentrated from bulk tissue culture preparations by ultrafiltration, using a GFE 15 fibre dialysing

membrane with a pore size of 10K (Dr. R.E. Randall, personal communication), and this presented the interesting option of employing the membrane for concentrating LTB-Pk.

In the following sections, both the ammonium sulphate precipitation and ultrafiltration protocols and the efficacy of each procedure at concentrating LTB-Pk from *Vibrio sp.*60 culture supernatants, is described.

B.2.1. Concentration and partial purification of LTB-Pk by ammonium sulphate precipitation

To determine whether ammonium sulphate precipitation could be employed for concentrating LTB-Pk, a 200ml culture of *Vibrio sp.*60 was grown at 30°C until the $O.D._{600}$ reached 0.4 and then LTB-Pk synthesis was induced by the addition of 0.05mM IPTG to the bacterial culture. After a 6 hour induction, the bacteria were pelleted by centrifugation and the 200ml culture supernatant retained. A 20 ml sample of the supernatant was mixed with $(NH_4)_2SO_4$ (to a saturation level of 50%), and the precipitated proteins were collected by centrifugation. Following resuspension in 5ml of PBS, excess salt was removed by dialysing the precipitated proteins extensively against the saline buffer.

15µl volumes from both the original crude preparation of LTB-Pk and the precipitated protein were electrophoresed through a SDS-polyacrylamide gel, and the protein bands stained with Coomassie blue, the result of which is shown in Fig.15.

Examination of the Coomassie stained gel revealed very few protein contaminants were detectable in the culture medium prior to precipitation, the two major proteins being LTB-Pk and a second protein of unknown function, which was present in higher quantities than LTB-Pk (lane 1). When the proteins present in the sample from the $(NH_4)_2SO_4$ precipitated fraction was examined, although both LTB-Pk and this second protein could be precipitated, the efficiency of their precipitation using a salt saturation level of 50%, varied remarkably. Whereas LTB-Pk could be readily precipitated and concentrated approximately 3-fold, it was apparent that the second protein precipitated weakly and thus, was not efficiently concentrated (lane 2). In addition, there was little

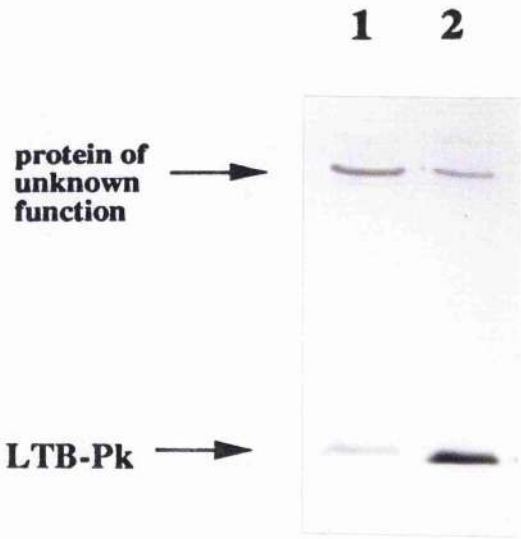


Figure 15. Ammonium sulphate precipitation of LTB-Pk from *Vibrio sp.60* cultures.
Coomassie blue stained gel comparing the concentration and purity of LTB-Pk from IPTG induced *Vibrio sp.* 60 culture supernatant before (lane 1) and after (lane 2) precipitation with ammonium sulphate at a final saturation level of 50%. The precipitated protein was resuspended in, and dialysed against PBS, to remove excess salt, prior to analysis. The final volume of concentrated material was less than the starting medium.

Figure 16. Cleavage of the Pk-tag from LTB-Pk following ammonium sulphate precipitation.
An equal sample of unprecipitated (lane 1) and precipitated (lane 2) LTB-Pk was electroblotted onto nitrocellulose paper and the presence of the Pk-tag determined in Western blot assays, by probing the blot with MAb SV5-P-k. Bound antibody was detected with HRP-conjugated Protein A, ECL and autoradiography.

evidence that any other proteins were precipitated/concentrated under the conditions described. These findings suggest that not only can $(NH_4)_2SO_4$ precipitation efficiently concentrate LTB-Pk, the procedure can also help to partially purify the fusion protein from the major protein contaminants in *Vibrio sp.*60 cultures.

However, closer examination of the Coomassie blue stained gel, revealed that precipitated LTB-Pk migrated to a position slightly lower than that of unprecipitated LTB-Pk, suggesting that the Pk epitope may have been cleaved from the enterotoxin subunit following precipitation. To determine if this was the case, 15µl samples of unprecipitated and precipitated LTB-Pk were analysed for the presence of the Pk-epitope by Western blotting utilising MAb SV5-P-k as the detection antibody (Fig. 16).

Examination of the resulting autoradiograph, revealed that LTB-Pk was detectable as a single band in the unprecipitated fraction (lane 1), but was hardly detectable in the sample from the precipitated fraction (lane 2). This finding confirms that the Pk epitope was cleaved from LTB following exposure of the culture medium to $(NH_4)_2SO_4$.

B.2.2. EDTA inactivation of protease activity; protection of the Pk-fusion

Sensitive LTB-fusions can be degraded by metallo-like proteases secreted from *Vibrio sp.*60 (Dr. T.R. Hirst, personal communication). If such protease activity was responsible for the cleavage of the Pk epitope, then the addition of ethylenediaminetetra-acetic acid di-sodium salt (EDTA), a well-known inactivator of metallo-like proteases, might preserve the fusion. To determine whether EDTA could prevent cleavage of the Pk tag from LTB-Pk, the precipitation reaction described above was repeated, and after resuspending the precipitate in PBS, the solution was divided into two equal aliquots. The first aliquot was dialysed against PBS over a 2-72 hour period, the second, against PBS supplemented with 10mM EDTA and the stability of the LTB-Pk fusion at each time interval, was verified by the Western blotting (Fig.17). As a control, LTB-Pk from unprecipitated cultures was included in the assay.

LTB-Pk was readily detectable in the control sample from the unprecipitated cultures (lane1). However, althought LTB-Pk was present in precipitated cultures that had been dialysed against PBS for 2 hours (lane 2), by 24 hours of dialysis, very little intact protein was detectable (lane 3), confirming that the Pk-epitope is cleaved from LTB-Pk following exposure of the culture medium to $(NH_4)_2SO_4$. In contrast to this finding, when the precipitated protein was dialysed in the presence of 10mM EDTA, the intact fusion protein was clearly detectable by MAb SV5-P-k at all times over the 72 hour dialysis period (lanes 6-9).

To confirm the efficacy of EDTA in preventing the cleavage of the Pk-epitope from LTB, a 15µl sample of precipitated LTB-Pk that had been dialysed in the presence of EDTA, was incubated with either *St.aureus*-saturated with MAb-SV5-P-k, or *St.aureus* alone. The proteins present in both the precipitated and non-precipitated fractions were subsequently electrophoresed through a SDS-polyacrylamide gel.

Coomassie blue staining of the SDS-polyacrylamide gel (Fig.18) revealed the $(NH_4)_2SO_4$ concentrated sample of LTB-Pk, that had been dialysed in the presence of EDTA, efficiently precipitated onto *St. aureus* when the bacteria were saturated with MAb SV5-P-k (lane 1), there being no detectable LTB-Pk degradation products in the unprecipitated supernatant (lane 2). Confirmation that this immune-precipitation of LTB-Pk was through the affinity of the intact fusion protein for the Pk-specific MAb, was provided when it was demonstrated that LTB-Pk did not precipitate onto the bacteria in the absence of the MAb SV5-P-k (lane 3) and thus, was detectable in the unprecipitated fraction (lane 4).

These results clearly demonstrate that EDTA is highly efficient at stabilising the LTB-Pk fusion after $(NH_4)_2SO_4$ precipitation, possibly through its ability to inactivate a metallo-activated protease secreted from *Vibrio sp.* 60.

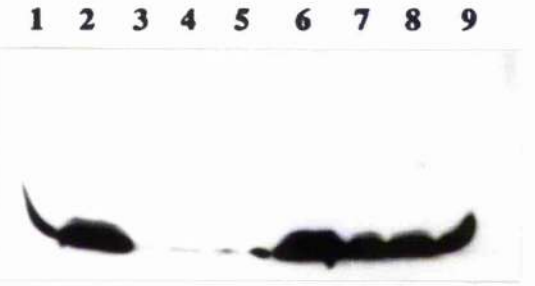


Figure 17 . Stability of LTB-Pk following ammonium sulphate precipitation and dialysis against EDTA.

LTB-Pk was precipitated from IPTG-induced *Vibrio sp.*60 culture medium with ammonium sulphate, then dialysed against PBS (lanes 2-5) or PBS supplemented with 10mM EDTA (lanes 6-9) for 2-72 hours, and the stability of the LTB-Pk fusion determined by Western blot assay.
Lane 1: unprecipitated LTB-Pk; lanes 2 & 6: 2 hour dialysis; lanes 3 & 7: 24 hour dialysis; lanes 4 & 8: 48 hour dialysis; lanes 5 & 9: 72 hour dialysis.

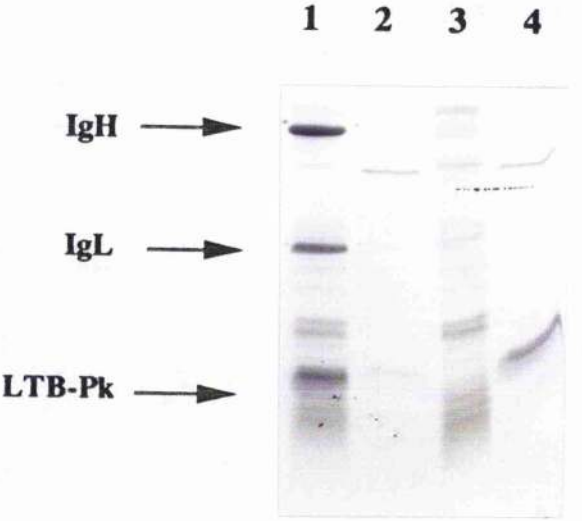


Figure 18. Immune-precipitation of LTB-Pk.

A sample of precipitated LTB-Pk that had been dialysed in the presence of 10mM EDTA was incubated with MAb SV5-P-k-saturated *St. aureus* (lanes 1&2) or *St.aureus* alone (lanes 3&4) and the polypeptides present in both the immune-precipitated complex (lanes 1&3) and the unprecipitated retenate (lanes 2&4) were separated by electrophoresis through a 15% SDS-polyacrylamide gel, which was stained with Coomassie blue.

B.2.3. Concentration of LTB-Pk by ultrafiltration.

The procedure for concentrating LTB-Pk by ultrafiltration using the GFE 15 fibre dialysing membrane was adapted from the protocol used to concentrate monoclonal antibodies from bulk tissue culture solutions.

A four litre culture of *Vibrio sp.*60 was induced with 0.05mM IPTG for 6 hours at 30°C, and the bacteria were separated from the culture medium by centrifugation. The resulting 4 litre supernatant was then applied to the GF15 dialysis membrane and the solution constantly recirculated through the dialysis unit under a pressure of 5-10 lbs/in^2 [Fig. 7(a)]. Such a strategy helped remove molecules that were smaller than the 10K pores of the membrane, as well as water molecules, resulting in a 150ml retentate which was dialysed *in situ* [Fig. 7(b)] against an appropriate buffer, before being recovered from the dialysis unit.

Figure 20 [Panels (a-c) lanes 1] show examples of the proteins that are concentrated following ultrafiltration and gives the clearest evidence yet that few protein contaminants are present in culture medium from which LTB-Pk has to be purified. In addition, estimation of the concentration of LTB-Pk in the ultrafiltrate in comparison to the original culture medium, suggested that approximately 85-90% of LTB-Pk was recovered from the membrane. Furthermore, since no extraneous salt agent was added at any time during the ultrafiltration process, it seemed unlikely that the Pk epitope would be cleaved from LTB and this was vindicated when it was demonstrated that LTB-Pk, concentrated by ultrafiltration, could be efficiently precipitated on *St. aureus* through the interaction of the anti-Pk MAb and the Pk epitope (data not shown).

These results provide evidence that, not only is ultrafiltration using a GFE 15 fibre dialysis membrane an excellent strategy for concentrating LTB-Pk proteins from large bulk *Vibrio sp. 60* cultures, but also, in terms of stability of the LTB-Pk fusion, it is better than ammonium sulphate precipitation.

B.3. Purification of LTB-Pk

Initial attempts to purify concentrated LTB-Pk by affinity chromatography using sepharose-based beads was unsuccessful. This was primarily due to the high affinity that the LTB has for sugar-based matrices [Finkelstein and Clements, 1979], such as sephadex, which required strong denaturation conditions to elute bound LTB-Pk. While the search for an alternative column support was underway, a report was published [Amin and Hirst, 1994], that demonstrated LTB could be purified from *Vibrio sp.*60 culture supernatants by hydrophobic interaction chromatography, using a phenyl superose support operated on a Fast Protein Liquid Chromatography (FPLC) system. This presented the possibility that hydrophobic interaction chromatography could be adapted for purifying LTB-Pk. Two hydrophobic supports were investigated; the phenyl superose matrix originally employed by Amin and Hirst to purify LTB, and a Macro-prep® t-butyl matrix (acrylamide-based beads), which offered a cheaper alternative to phenyl superose, and did not necessitate the need for specialised equipment. The use of ion-exchange chromatography, using acrylamide-based beads, was also examined.

B.3.1. Purification of LTB-Pk using a phenyl superose support and FPLC.

The procedure outlined below is a modification of the method described by Amin and Hirst [1994].

A 4 litre volume of *Vibrio sp.*60 that had been induced with IPTG for 6 hours was centrifuged to pellet the bacteria, and the supernatant concentrated by the ultrafiltration method described above. The protein-rich filtrate was dialysed *in situ* against 20 volumes of 20mM Tris-HCl, 10mM EDTA, pH 7.5 and the resulting 150 ml dialysate recovered from the membrane. A 20ml volume of the retentate was mixed with solid ammonium sulphate to a concentration of 30% saturation, then clarified by centrifugation at 17,400g. The supernatant was retained, 5ml of which was filtered through a 0.22μm filter and applied to a 1ml phenyl superose column, that had previously been equilibrated with 20mM Tris-HCl pH 7.5, 10mM EDTA containing 1M $(NH_4)_2SO_4$, on an FPLC system.

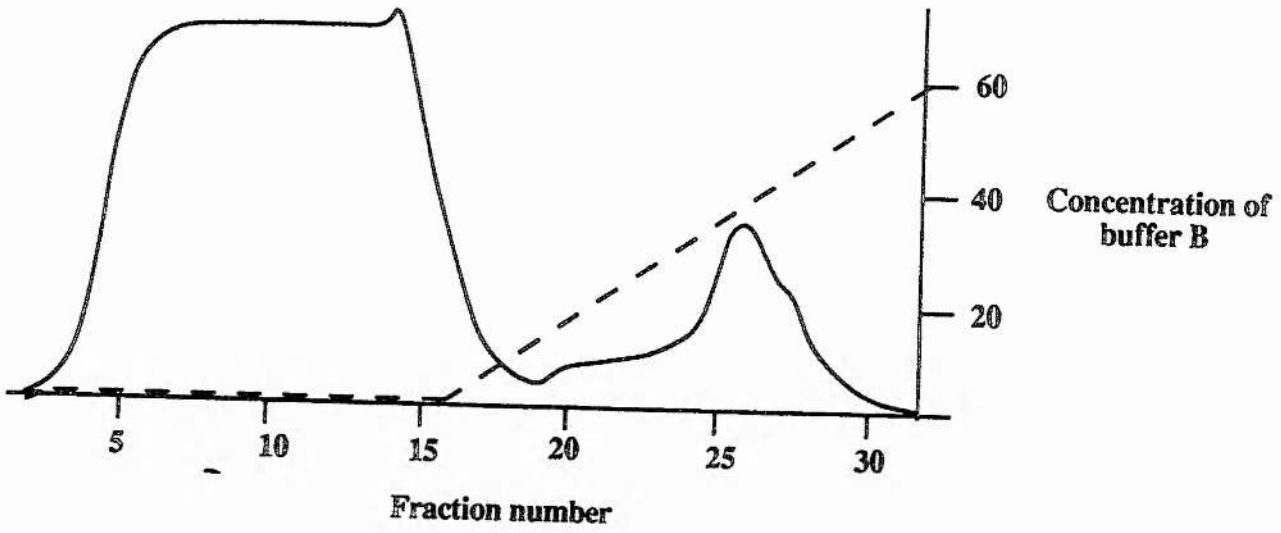


Figure 19. Protein purification profile by phenyl superose hydrophobic chromatography. The figure shows an example of the optical density measured at 280nm during the purification of LTB-Pk from concentrated *Vibrio sp.* 60 culture medium, using the phenyl superose hydrophobic matrix operated on an FPLC system. A 5ml sample of ultrafiltrated *Vibrio sp*60 culture medium was applied to a phenyl superose column and the column was washed in 20mM Tris-HCl, pH 7.5, 10mM EDTA containing 1M ammonium sulphate (Buffer A). A decreasing salt gradient was then generated by the application of Buffer B (20mM Tris-HCl, pH 7.5, 10mM EDTA). 1ml samples were collected throughout the purification (X-axis) and the O.D. at 280nm monitored. Fractions 1-15; material that fails to bind the column, fractions 20-28; eluted material.
N.B.the (— — —) line represents the salt concentration of the 20mM Tris-HCl, 10mM EDTA buffer.

Once the sample was loaded the column was washed in the above buffer to remove unbound proteins, and purified material was eluted by a decreasing salt gradient. Throughout the purification procedure, 1ml fractions were collected and the presence of proteinaceous material monitored by measuring the adsorbance at 280nm. An example of the phenyl superose purification profile is shown in Fig.19.

At two stages during the purification process, high levels of materials that adsorbed strongly at 280 nm were detectable; 1) during early washing steps, representing protein that failed to bind the column (fractions 1-15) and 2) during the elution step, at an ammonium sulphate molarity of approximately 0.65M (fractions 25-28). To further investigate the proteins present in these fractions, fractions 1-15 were pooled, as were fractions 25-28, and 15µl samples were removed, electrophoresed through a SDS-polyacrylamide gel and the proteins visualised by Coomassie blue staining [Fig.20 , Panel (a)].

Coomassie blue staining of the polypeptide chains, revealed that of the original proteins present in the unpurified material (lane 1), the vast majority of the contaminating proteins failed to bind to the column and were removed during the washing steps, (lane 2). In contrast to this finding, it was evident that the LTB-Pk efficiently bound to the phenyl superose, and was eluted at a salt molarity of approximately 0.65 (lane 3). Estimation of the concentration of LTB-Pk applied to the phenyl superose, in comparison to the quantity of LTB-Pk eluted from the column, suggested a recovery rate of approximately 67%. However it was noteable that, traces of the major contaminating protein could still be detected in LTB-Pk that had been purified in this manner.

B.3.3. Purification of LTB-Pk by hydrophobic chromatography using a Macro-prep.® t-butyl support.

The second hydrophobic column to be investigated was a Macro-prep.® t-butyl support. The criteria for purifying LTB-Pk on this mildly hydrophobic matrix, was an adaptation of the procedure for the purification of LTB-Pk on phenyl superose.

A 40 ml sample of the protein-rich dialysate containing concentrated LTB-Pk was applied under high salt conditions, to a 5ml t-butyl column that had been equilibrated with 20mM Tris-HCl, pH 7.5, 10mM EDTA containing 1M $(NH_4)_2SO_4$ at room temperature. Once all the protein solution had been added, the column was washed in 10 column volumes of 20mM Tris-HCl, pH 7.5, 10mM EDTA containing 1M $(NH_4)_2SO_4$ to remove unbound proteins. A decreasing ammonium sulphate concentration step gradient was generated by washing the column in 200ml of 20mM Tris-HCl, pH 7.5, 10mM EDTA containing 1M, 0.75M, 0.5M, and 0.25M $(NH_4)_2SO_4$ sequentially. Throughout the purification process 5ml fractions were collected and a 1µl sample from each fraction was allowed to adsorb onto nitrocellulose paper. The presence of protein in each fraction was visualised by staining the nitrocellulose with 0.2M Naphthalene black (data not shown). By examining the amount of stain adsorbed by the protein spots, it was evident that proteins that failed to bind to the t-butyl matrix were detectable in fractions collected during the early stages of the purification procedure (data not shown). In subsequently collected fractions, the protein levels detectable fell, until the buffer containing 0.75M ammonium sulphate was applied to the column (data not shown). Thereafter, the level of protein eluting from the column fell in correlation with a decrease in the ammonium sulphate concentration.

Fractions collected early in the purification, that contained the highest levels of protein, were pooled, as were the fractions that were assumed to contain high levels of eluted protein. 15µl volumes from both samples were subjected to SDS-PAGE and the resulting polypeptide chains stained with Coomassie blue.

The results presented in Fig.20, Panel (b), demonstrate that purification of LTB-Pk using the t-butyl hydrophobic support gave a similar result to that of the phenyl superose column, where the majority of contaminating proteins present in the unpurified sample (lane 1) were removed during the washing steps (lane 2). Furthermore, LTB-Pk efficiently bound to the t-butyl support (lane 2) but was eluted following the application of a decreasing salt gradient to the column (lane 3). Estimation of the recovery rate of LTB-

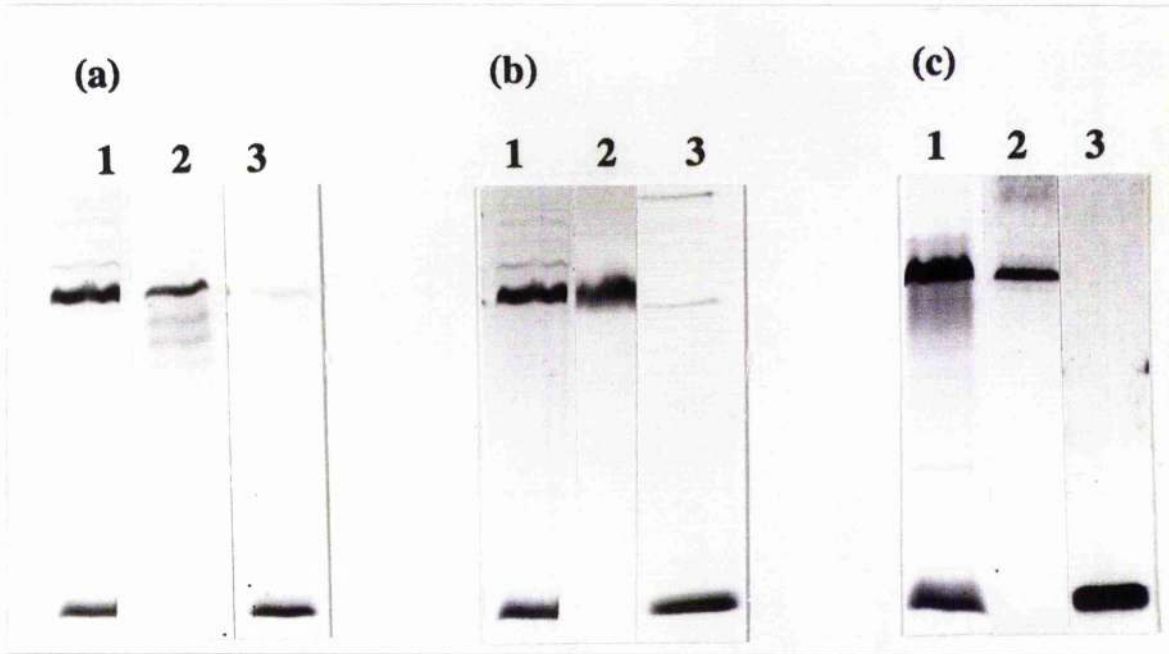


Figure 20. Purification of LTB-Pk.
An example of the efficacy of Phenyl superose (Panel (a)), Macroprep® t-butyl (Panel (b)) or Bio-rex-70 (Panel (c)) affinity columns in purifying LTB-Pk from *Vibrio sp.*60 culture medium. IPTG-induced *Vibrio sp.* 60 culture medium was concentrated by ultrafiltration, and a sample of the concentrated solution applied to the appropriate column. The proteins present in 15µl samples from the concentrated starting material (lane 1), were compared to the proteins present in a similar volume from the column wash (lane 2) and the column eluate (lane 3) by 15% (w/v) SDS-PAGE and Coomassie blue staining.

Pk from the column revealed that, of the starting material that was applied to the column, 62% of LTB-Pk eluted.

However, once again, it was evident that LTB-Pk was not completely purified, since there was evidence of minor traces of contaminating proteins in the eluted material (lane 3)

B.3.3. Purification of LTB-Pk by ion-exchange chromatography using a Bio-Rex® cation-exchange support.

The third method used for purifying LTB-Pk utilised a sulphur-based cation-exchange column based on acrylamide-bead support. Preliminary experiments established that in a buffer composed of 0.25mM Na_2PO_4, pH 7.0, purified LTB molecules (a kind gift from Dr. T.R. Hirst) were sufficiently ionised to enable the protein to bind to the negatively charged support, but could be eluted following the addition of 25mM Na_2PO_4, 0.5M NaCl, pH 7.5 to the column. It was decided to assess the ion-exchange protocol for its ability to purify LTB-Pk from *Vibrio sp.* 60 culture medium.

A 4 litre culture of *Vibrio sp.* 60 was grown at 30°C and when the $O.D._{600}$ reached 0.4, LTB-Pk was induced for 6 hours by the addition of 0.05mM IPTG to the bacterial culture. The bacteria were pelleted by centrifugation, and the 4 litre supernatant was concentrated by ultrafiltration, as previously described. When the supernatant was concentrated to a 150ml volume, the solution was dialysed *in situ* against 25mM Na_2PO_4, 10mM EDTA, pH 7.0, clarified by centrifugation at 17,400g and the supernatant applied directly to a 25ml Bio-rex® 70 cation-exchange column that had been equilibrated with 25mM Na_2PO_4, 10mM EDTA, pH 7.0. The column was washed with 10 volumes of 25mM Na_2PO_4, 10mM EDTA, pH 7.0 to remove any unbound proteins and bound protein was then eluted by the application of a buffer composed of 25mM Na_2PO_4, 10mM EDTA, 0.5M NaCl, pH 7.5 to the column. Samples were taken throughout the purification process and assayed for the presence of protein material using the Naphthalene black-based procedure described above. The fractions containing protein that failed to bind the column, were pooled, as were the fractions that contained protein that

eluted from the column and 15 µl samples were removed for analysis by SDS-PAGE and Coomassie blue staining [Fig. 20, Panel (c)].

It was evident that of the major proteins present in the original concentrated material (lane 1) LTB-Pk bound to the ion-exchange column at pH 7.0, [whereas the major protein contaminants did not (lane 2)], but was eluted after the addition of 25mM Na_2PO_4, 10mM EDTA, 0.5M NaCl, pH 7.5 buffer to the column (lane 3). Of more significance, LTB-Pk was the only protein detectable in the eluted material, all the other contaminating proteins being removed during the washing steps of the purification process (lane 2). Thus, in terms of protein purity, ion-exchange chromatography is more efficient at purifying LTB-Pk from concentrated culture medium, than hydrophobic chromatography. Furthermore, estimation of the yield of purified LTB-Pk from the cation-exchange matrix, suggested approximately 84% of LTB-Pk eluted.

B.4. Functional properties of LTB-Pk purified by ion-exchange chromatography and the stability of the Pk-epitope.

Examination of the proteins eluted from the three columns investigated in the previous sections, demonstrated that, ion-exchange chromatography was the best and most convenient method for purifying LTB-Pk. To confirm that purified LTB-Pk retained the physiological properties of unpurified LTB-Pk, the ability of purified LTB-Pk to oligomerize and bind to GM1-ganglioside were investigated *in vitro*.

Firstly, the oligomerization properties of purified LTB-Pk were determined by analysing the migration pattern of heat-treated and untreated samples of the fusion protein, following SDS-PAGE. As a comparison, purified LTB molecules were also included in the assay. Coomassie blue staining of the resulting polypeptide bands revealed that, in boiled samples purified LTB and LTB-Pk migrated as monomers (Fig.21, lanes 1 & 2, respectively), LTB-Pk migrating to a position slightly higher than LTB. When purified LTB and LTB-Pk were applied to the SDS-polyacrylamide gel without prior heat-treatment, both proteins migrated as stable oligomers (Fig. lanes 3 & 4, respectively). Secondly, the affinity of purified LTB-Pk for GM1-ganglioside was

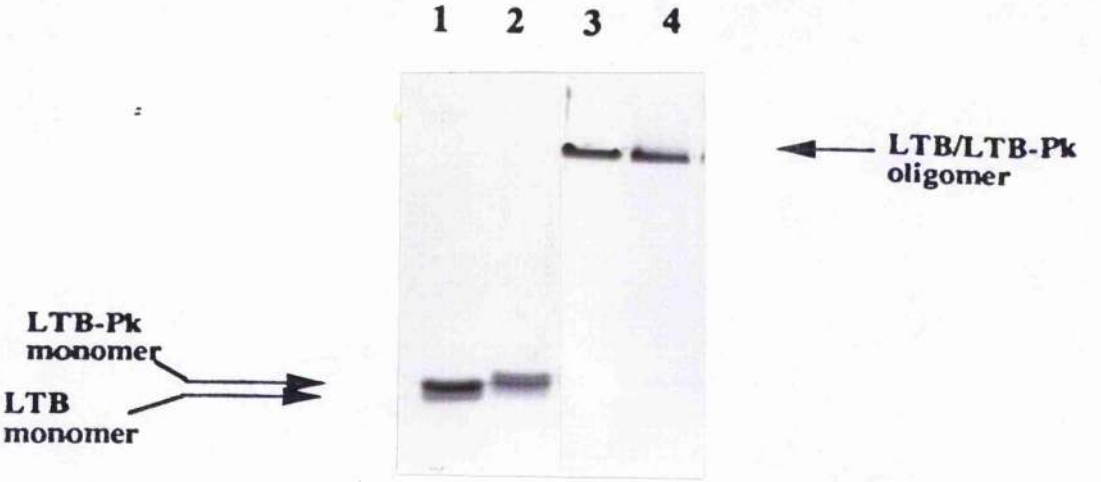


Figure 21. Oligomerization of LTB-Pk following purification by ion-exchange chromatography

Samples of purified LTB (lanes 1 & 3) and purified LTB-Pk (lanes 2 & 4) were mixed with an appropriated volume of 4X disruption buffer and either boiled for 5 minutes (lanes 1 & 2) or left at room temperature (lanes 3 & 4) prior to SDS-PAGE on an 15% (w/v) SDS-polyacrylamide gel. The resulting polypeptide chains were visualized by Coomassie blue staining.

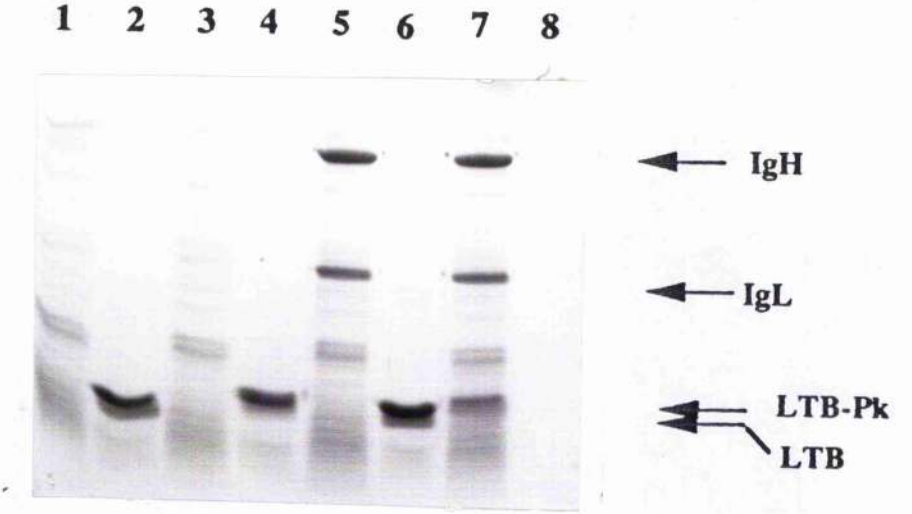


Figure 23. Immune-precipitation of purified LTB-Pk.

The stability of the LTB-Pk fusion after purification of LTB-Pk by ion-exchange chromatography was examined in immune-precipitation assays. Samples of purified LTB or LTB-Pk were were incubated with either *St. aureus* alone (lanes 1-4) or MAb SV5-P-k-saturated *St. aureus* (lanes 5 & 8) and both the precipitates (lanes 1,3,5 & 7) and the unprecipiated supernatants (lanes 2,4,6 & 8) were analysed by 18% SDS-PAGE and Coomassie blu staining for the presence of purified LTB (lanes 1,2,5 & 6) and LTB-Pk (lane 3, 4, 7 & 8)

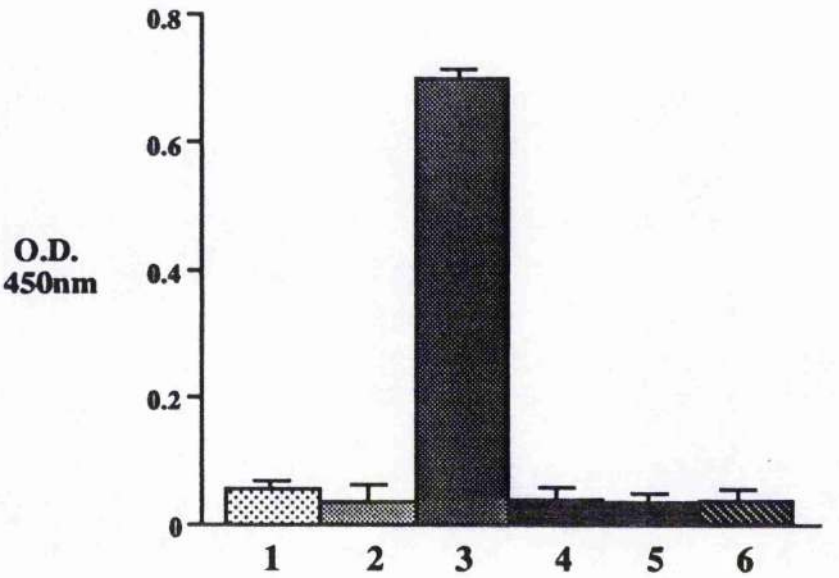


Figure 22. Binding of purified LTB-Pk to GM1-ganglioside.
The figure shows the retention of the GM1-binding property of LTB-Pk after purification by ion-exchange chromatography. 96-well microtitre plates were coated with either (1.5μg/ml) GM1(lanes 1-3) or (1mg/ml) ovalbumin (lanes 4-6) and then incubated with PBS alone (lanes 1&4), MAb SV5-P-k (lanes 2&5) or purified LTB-Pk (lanes 3&6). The anti-Pk MAb was added to all wells and bound protein was detected with HRP-conjugated Protein A. Following addition of the HRP-substrate, the adsorbance at 450nm was measured.
The results are presented as the mean ± standard deviation for duplicate cultures.

vindicated in a GM1-ELISA assay (Fig.22) where it was evident that LTB-Pk bound to the ganglioside in a highly specific manner (column 3). Thus, the purification of LTB-Pk by ion-exchange chromatography does not destroy the physiological properties of LTB-Pk.

In addition to the analysis of the physiological properties of purified LTB-Pk, the stability of the Pk-fusion following purification was determined in immune-precipitation reactions, where both purified LTB and LTB-Pk were assessed for their ability to bind to MAb-SV5-P-k-saturated *St. aureus*, or to *St. aureus* alone.

As shown in Fig. 23, purified LTB was unable to bind to either *St.aureus* alone (lane 1) or anti-Pk MAb-saturated *St.aureus* (lane 5) and was therefore, detectable in the unprecipitated fractions (lanes 2 and 6). Likewise, purified LTB-Pk did not bind to *St. aureus* alone (lane 3) and was also detectable in the unprecipitated fraction (lane 4). In contrast to this finding, when purified LTB-Pk was mixed with MAb SV5-P-k-saturated *St.aureus*, all the purified protein was present in the precipitate (lane 7), with no evidence of degraded LTB-Pk products in the unprecipitated fraction (lane 8). Thus, purification of LTB-Pk by ion-exchange chromatography does not result in the destruction of the Pk-epitope.

SECTION C. Expression and Purification of His-SIV-Pk Proteins and Construction of SIV-MAb-LTB complexes.

C.1. Introduction.

In section C of the results, the recombinant SIV proteins used in the immunisations procedures, are introduced and their unique tag-linked purification by nickel affinity chromatography is described. In the later stages of the section, the construction of the novel SIV-MAb-LTB immunogen, and the targeting of such complexes onto GM1-ganglioside, is presented in detail.

C.2. Construction and expression of recombinant His-SIV-Pk proteins.

The addition of the 14 amino acid Pk tag to the C-termini of a variety of Simian Immunodeficiency Virus proteins has been described in detail by Hanke and colleagues [Hanke *et al.*,1994]. Such SIV-Pk proteins were expressed as fusion proteins with a 12 amino acid long peptide containing six histidines (His) at the N-terminal of the recombinant proteins, in the *E.coli* pQE-9-derived vector [Randall *et al.*, 1993]. In this expression system, the novel His-SIV-Pk gene is under control of the *lac* promoter, thus, the recombinant proteins can be induced by IPTG. The choice of recombinant SIV gag gene products, His-p17-Pk and His-p27-Pk, for the vaccination strategies described later, was motivated by the moderate to high level of expression of these recombinant proteins in *E.coli* (10mg/litre and 200-700μg/litre, respectively), and that both antigens have been shown to be immunogenic when incorporated into solid matrix antibody-antigen complexes [Randall *et al*, 1994].

C.2. Purification of His-SIV-Pk proteins by Nickel Affinity Chromatography.

The protocol developed for the purification of His-SIV-Pk antigens was based on the observation that histidine residues have a high affinity for metal cations, and can, therefore, be purified by chelating immobilised metal ions such as nickel [Smith *et al.*, 1988].

1 litre cultures of *E.coli* containing plasmids pQE917Pk, encoding His-p17-Pk, or pQ927Pk, encoding His-p27-Pk, were induced at 26°C with 0.05mM IPTG when the $O.D._{600}$ reached approximately 0.4-0.6. After 2-3 hour inductions, the bacteria were harvested by centrifugation, resuspended in 30ml of 20mM Tris-HCl, 0.3M NaCl, pH 8.0 and the intracellular proteins released by lysozyme treatment, followed by vigorous sonication. The protein rich lysate was centrifuged, and the resulting supernatant incubated with a 1ml sample of Ni^{2+}-NTA resin for 1 hour at 4°C. The beads were loaded into a column and unbound proteins were removed by washing the column in 20mM Tris-HCl, 0.3M NaCl, pH 8.0. Bound His-SIV-Pk proteins were eluted by the application of

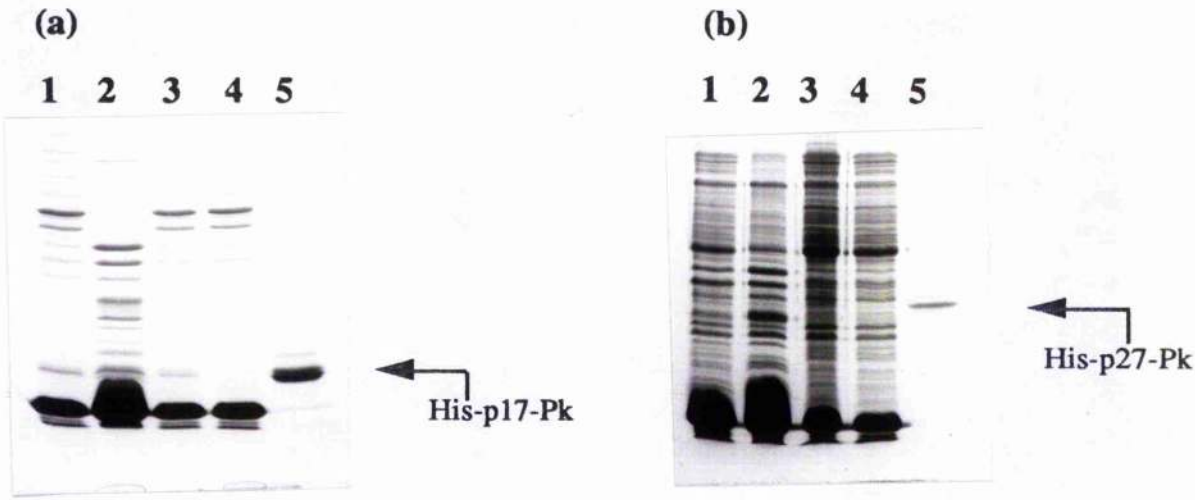


Figure 24. Purification of the His-SIV-Pk antigens.
E.coli containing plasmids that encode the recombinant His-p17-Pk (Panel (a)) and His-p27-Pk (Panel (b)) proteins were induced for 3 hours with 0.05mM IPTG at 26°C. The His-SIV-Pk antigens were purified by Nickel affinity chromatograpy and the purity of the eluted protein was examined by 15 % (w/v/) SDS-PAGE and Coomassie blue staining.
Lanes 1;total cell lysate, lanes 2; insoluble cell fractions, lanes 3; soluble cell fractions, lanes 4; protein that did not bind to the nickel columns and lanes 5; protein that was eluted from the nickel columns with imidazole (see text).

250mM Imidazole, 20mM Tris-HCl, 0.3M NaCl, pH 7.4 to the column and the purity of the eluted His-p17-Pk and His-p27-Pk, were examined by SDS-polyacrylamide gel analysis [Fig. 24, Panels (a) & (b), respectively].

Coomassie blue staining of the polyacrylamide gels revealed that the His-SIV-Pk proteins are present in the soluble fraction upon lysis (lanes 3) and are not restricted to insoluble inclusion bodies (lanes 2) in *E.coli.*, a finding that supports the observations of Dr. T. Hanke [research thesis, 1993]. The vast majority of the contaminating material was removed during the washing stages (lanes 4) yielding a highly purified His-SIV-Pk protein upon elution (lanes 5).

C.3. Construction of the Simian Immunodeficiency Virus-Antibody-LTB (SIV-MAb-LTB) Complex.

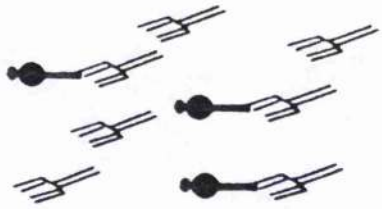
The method that was developed to produce SIV-MAb-LTB complexes can be divided into three major steps; 1) incubation of purified His-SIV-Pk with a five-fold excess of purified MAb SV5-P-k, such that, on average a single antibody molecule will only have one molecule of antigen attached 2) purification of SIV-MAb complexes from uncomplexed antibody, by nickel affinity chromatography, and 3) linkage of the SIV-MAb complexes to LTB-Pk via the free antigen binding site on the antibody molecule. A diagrammatic representation of the procedure is shown in Fig. 25.

(1) Construction of SIV-MAb complexes

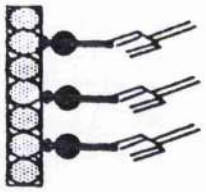
The formation of immune-complexes that contained an His-SIV-Pk antigen occupying only one of the antigen-binding sites of MAb SV5-P-k, thereby leaving the second site free for complexing with LTB-Pk, was the most critical step in the construction of SIV-MAb-LTB complexes. It is generally accepted that in a solution in which the concentration of an antibody molecule is in excess to its specific antigen, the vast majority of antibody molecules will have only one of their antigen-binding sites occupied by the antigen. On this basis, it was thought that mixing His-SIV-Pk proteins and MAb SV5-P-k at a molar ratio of 1:5 (SIV:MAb) would result in sub-saturation of the anti-Pk MAb. Thus, as a first step in the construction of SIV-MAb-LTB complexes, the concentration of purified His-SIV-Pk in relation to the concentration of purified MAb SV5-P-k was estimated by SDS-PAGE and Coomassie blue staining (data not shown).

(2) Purification of SIV-MAb complexes.

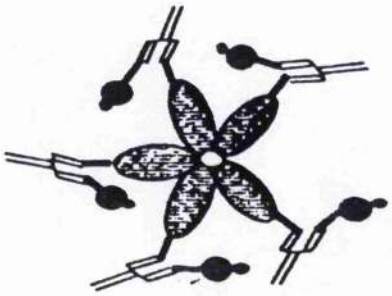
The second step in the construction of SIV-MAb-LTB complexes involved the removal of uncomplexed antibody. After incubating His-SIV-Pk and MAb SV5-P-k at a molar ratio of 1:5 for 50 minutes at 4°C, the solution was mixed with a 1ml volume of Ni^{2+}-NTA resin for 45 minutes at 4°C. After loading the resin into a column, uncomplexed MAb SV5-P-k that did not bind to the Ni^{2+}-NTA resin, was removed by washing the column in 20 volumes of 20mM Tris-HCl, 0.3M NaCl, pH 8.0. Purified SIV-MAb complexes were then eluted by the addition of 0.25M Imidazole, 20mM Tris-HCl, 0.3M NaCl, pH 7.4 to



Step One.
Pk-linked SIV proteins containing a six histidine tag at the N-terminus is mixed with Pk-specific MAb SV5-P-k, at a SIV:MAb ratio of 1:5.
This ensure incomplete saturation of the MAb.



Step Two
The affinity of the histidine tag for chelating ions enables SIV-MAb complexes to be purified by nickel affinity chromatography, and faciliates the removal of excess MAb.



Step Three
Quantitation of the amount of SIV antigen incorporated into the immune-complex by SDS-PAGE, determines of the concentration of LTB-Pk that will suffice to fill the remaining antigen-binding site on the MAb.

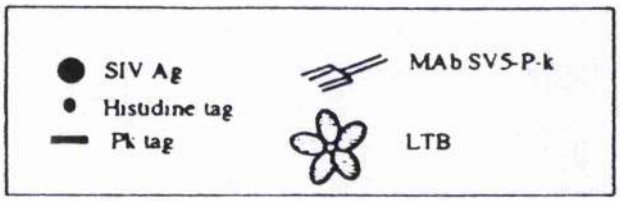


Figure 25. Construction of SIV-MAb-LTB complexes.
A diagrammatic representation of the three step procedure involved in the construction of SIV-MAb-LTB complexes.

the column. To estimate if these eluted SIV-MAb complexes had an His-SIV-Pk protein occupying only one antigen-binding site of MAb SV5-P-k, the concentration of the His-SIV-Pk protein in relation to the concentration of the light chain of MAb SV5-P-k, was analysed by SDS-PAGE and Coomassie blue staining. An example of such an analysis is shown in Figure 26. It is evident from the Coomassie stained gel, that the concentration of His-p27-Pk is approximately half the concentration of the light chain of the anti-Pk MAb, suggesting that the antigen-binding sites of MAb SV5-P-k are incompletely saturated.

(3) Linkage of LTB-Pk to purified SIV-MAb complexes.

The last step in the construction of SIV-MAb-LTB complexes involved the linkage of purified SIV-MAb complexes to LTB-Pk. Since only one antigen-binding site of MAb SV5-P-k was presumed to be unoccupied, it was thought that mixing SIV-MAb complexes with LTB-Pk at a molar ratio of 1:1, would suffice to saturate the remaining antigen-binding site of MAb SV5-P-k. These resulting SIV-MAb-LTB complexes would thereby contain an identical amount of His-SIV-Pk and LTB-Pk proteins. Thus, the concentration of LTB-Pk in relation to the concentration of His-SIV-Pk in SIV-MAb complexes was estimated by SDS-PAGE and Coomassie blue staining. By comparing the strengths of the Coomassie stained bands, the amount of LTB-Pk that gave a signal identical to that of the His-SIV-Pk protein, determined the amount of LTB-Pk that was to be mixed with the immune complex.

To establish whether mixing SIV-MAb complexes with LTB-Pk at a molar ratio of 1:1 resulted in all the available LTB-Pk being incorporated into the complex, an immune precipitation assay was performed. SIV-MAb-LTB complexes were mixed with *St. aureus* and after a 30min incubation, the precipitates were pelleted by centrifugation. Both the precipitated pellet, and the unprecipitated supernatant were analysed by SDS-PAGE and Coomassie staining. Such analysis demonstrated that LTB-Pk efficiently bound to the SIV-MAb complex and was present in the precipitated fraction, there being no detectable free LTB-Pk molecules present in the unprecipitated supernatant (data not shown).

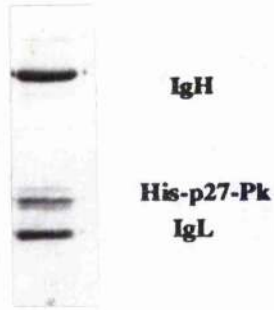


Figure 26. Sub-saturation of MAb SV5-P-k.
The figure shows an example of SIV-MAb complexes that have been constructed by incubating recombinant p27 and MAb SV5-P-k at a ratio of 1:5, and then purified nickel affinity chromatography. A 15µl sample of the purified complexes was subjected to SDS-PAGE and the protein bands stained with Coomassie blue.

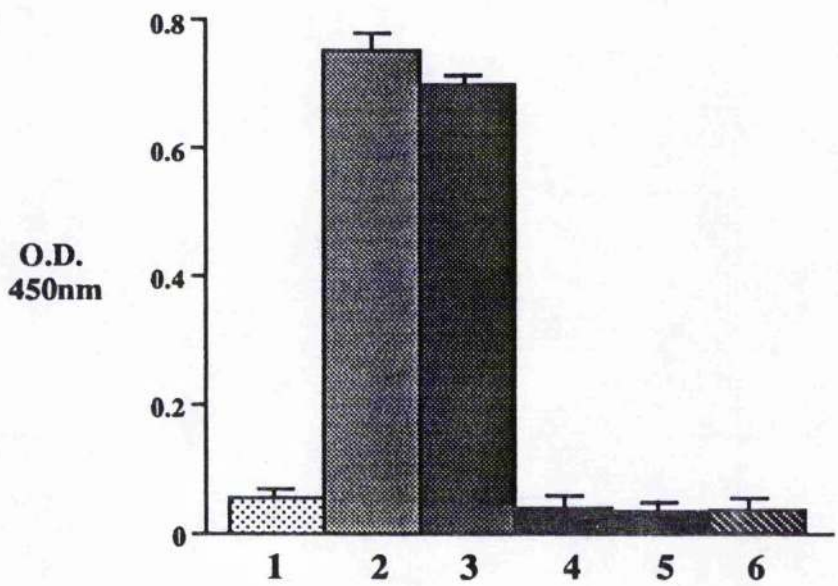


Figure 27. Binding of SIV-MAb-LTB complexes to GM1.
The figure shows the targeting to GM1-binding by SIV-MAb-LTB complexes. 96-well microtitre plates were coated with either (1.5µg/ml) GM1(lanes 1-3) or (1mg/ml) ovalbumin (lanes 4-6) and then incubated with SIV-MAb complexes (lanes 1&4), MAb-LTB complexes (lanes 2&5) or SIV-MAb-LTB complexes (lanes 3&6). Bound complexes were detected with HRP-conjugated Protein A. Following addition of the HRP-substrate, the adsorbance at 450nm was measured.
The results are presented as the mean ± standard deviation for duplicate cultures.

Thus, mixing SIV-MAb complexes with LTB-Pk at a molar ratio of 1:1, results in all the available LTB-Pk molecules being incorporated into the immune-complexes.

C.4. Binding of the SIV-MAb-LTB complex to GM1-ganglioside.

To further analyse SIV-MAb-LTB complexes, the ability of such complexes to bind to GM1-ganglioside was investigated in a GM1-ELISA assay.

A 96-well microtitre plate was coated with 1.5μg/ml GM1-ganglioside or ovalbumin and then a 1:10 dilution of SIV-MAb-LTB complexes was added to the wells. As positive and negative controls, MAb-LTB and SIV-MAb complexes (respectively) were included in the assay. After a 1 hour incubation, bound complexes were detected with HRP-conjugated Protein A, substrate added and the adsorbance at 450nm measured (Fig. 27).

There was no evidence that SIV-MAb complexes were capable of binding to GM1 (lane 1) as shown by the weak adsorbance at 450nm. In contrast, both MAb-LTB (lane 2) and SIV-MAb-LTB (lane 3) bound to GM1 with high affinity, demonstrating that the incorporation of LTB-Pk into the MAb-SIV complex can bind the immune complex onto GM1-ganglioside *in vitro*.

SECTION D. Systemic Immunogenicity of LTB-Pk.

D.1. Introduction.

The mechanism(s) responsible for the adjuvant nature of LTB is, at present, unknown. However, the physiological properties of LTB i.e. its existence as a pentamer and affinity for GM1-ganglioside are thought to play a critical role. In addition, it is theorised that the high immunogenicity of LTB itself, may contribute to its adjuvancity [Dertzbaugh *et al.*, 1990]. In Section D, the immunogenicity of LTB-Pk following parental administration and the attempts to classify the antibody response to the recombinant enterotoxin subunit, are described.

It is important to note, that, at the time of these preliminary immunogenicity studies, an efficient purification protocol for LTB-Pk was unavailable. Thus, the vaccines contained LTB-Pk that had been precipitated from IPTG induced *Vibrio sp.*60 culture supernatants with ammonium sulphate, and then dialysed against PBS containing 10mM EDTA.

D.2. Systemic Immunogenicity of LTB-Pk.

Peptide epitopes have been linked to LTB in attempts to raise an immune response to the peptide [e.g. Schodel *et al.*, 1991]. It was of interest to monitor the immunogenicity of LTB-Pk, both in terms of the antibody response to LTB and to the Pk epitope. In addition, the immunogenicity of MAb-LTB complexes were also investigated, since it was of interest to establish whether the linkage of MAb SV5-P-k to LTB-Pk influenced the immunogenicity of the fusion protein, a factor that could have implications on the use of SIV-MAb-LTB complexes as immunogens.

Groups of four 6-8 week old Balb/c mice were immunised with the equivalent of 1μg LTB-Pk alone (Group A) or after adsorption of the fusion protein onto the adjuvant, alum (Group B). LTB-Pk was mixed with MAb SV5-P-k at a molar ratio of 1:1 and the equivalent of 1μg LTB-Pk in MAb-LTB complexes were injected intraperitoneally either alone (Group C), or after adsorption onto alum (Group D). Prior to immunisation, a 15μl sample of each vaccine was analysed by SDS-PAGE and Coomassie blue staining, to ascertain that each vaccine contained an equivalent amount of LTB-Pk (Fig.28).

All mice were immunised three times with their respective vaccines, two weeks separating the first and second vaccination, and three weeks the second and third. Ten days after the final immunisation, the sera from each mouse was assayed for antibodies specific for LTB-Pk, or a control antigen (ovalbumin) using an ECL-based immune assay.

Briefly, the sera from each mouse within a vaccination group was diluted 1:200 and from this starting dilution, the sera was further serially diluted 1:2 in the wells of terasaki plates. MAb SV5-Pk and sera from non-immunised mice were similarly treated and used as positive and negative regulatory controls (respectively) in the assay. The

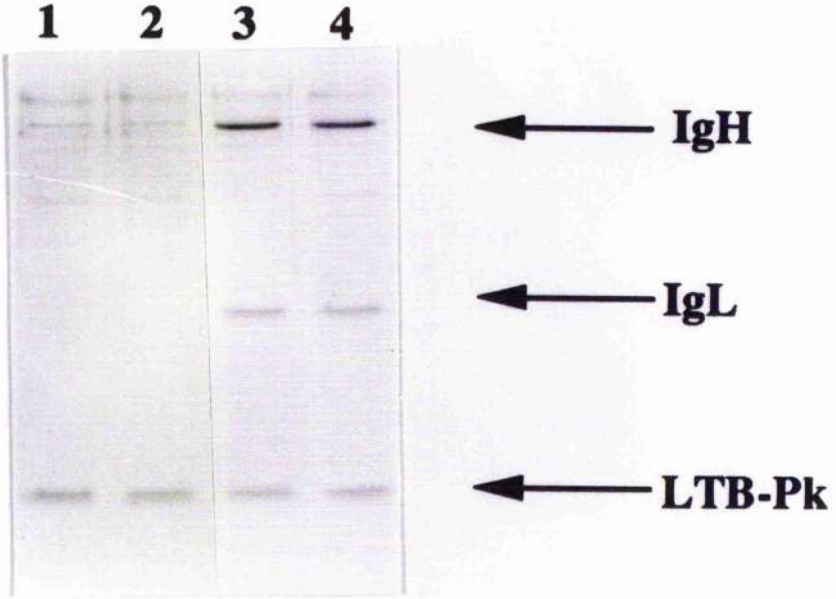


Figure 28. Comparative analysis of the LTB-Pk-based vaccines.
Analysis of the vaccines used for immunization against LTB-Pk. Each vaccine was resuspended to a final volume of 200µl in PBS and 30µl removed, electrophorised through a 15% SDS-polyacrylamide gel and the polypeptide chains stained with Coomassie blue. Lane1: soluble LTB-Pk; lane 2: LTB-Pk adsorbed onto alum; lane 3: LTB-Pk conjugated to MAb SV5-P-k (MAb-LTB); lane 4: MAb-LTB adsorbed onto alum.

terasaki plates were overlaid with individual nitrocellulose sheets that had been coated with either LTB-Pk or a control antigen (ovalbumin), and the plates were incubated for one hour at room temperature. Total antibody levels in the sera of vaccinated and control mice, to the respective antigens, were detected by incubating the nitrocellulose sheets with HRP-conjugated anti-mouse immunoglobulin (Ig), followed by exposure to ECL buffers and autoradiography. An example of the ECL-immune assay is shown in Fig.29 and the antibody titres to LTB-Pk for each vaccination group presented in Table 2. column 1.

Sera from non-immunised mice failed to bind to either of the target antigens, highlighting the specificity of the immune assay. Following three intraperitoneal vaccinations with LTB-Pk alone, all mice in Group A exhibited a high degree of immunity to LTB-Pk, with the average titre within the group being approximately 25,600. Interestingly, this immune response to LTB-Pk was not, on average, enhanced by administering LTB-Pk in association with alum (Group B). Although immunisation with MAb-LTB (Group C) resulted in a slight decrease in the immune response to LTB-Pk, this could be overcome by adsorbing the immune complex onto alum, prior to vaccination (Group D). These findings suggest that LTB-Pk itself, is a potent systemic immunogen, that does not apparently require the presence of additional adjuvants to potentiate the response to fusion protein. However, linkage of MAb SV5-P-k to LTB-Pk may slightly decrease the potency of LTB-Pk.

When the immune sera was re-assayed for antibodies capable of recognising the control antigen, ovalbumin, there was no evidence that any vaccinated mouse contained immunity to ovalbumin [Fig.29 Panel (b)].

To further analyse the immune response to LTB-Pk, the serum from each vaccinated mouse was assayed for antibodies specific for the Pk-epitope to investigate whether the antibodies, detectable to LTB-Pk, were interacting with the Pk-tag. The sera was diluted as before, then incubated with nitrocellulose sheets that had been previously coated with Glutathione-*S*-transferase-linked Pk (GST-Pk). Bound antibody was once again detected in ECL-immune assays that utilised an HRP-conjugated anti-mouse immunoglobulin. The antibody titres are shown in Table 2, column 2.

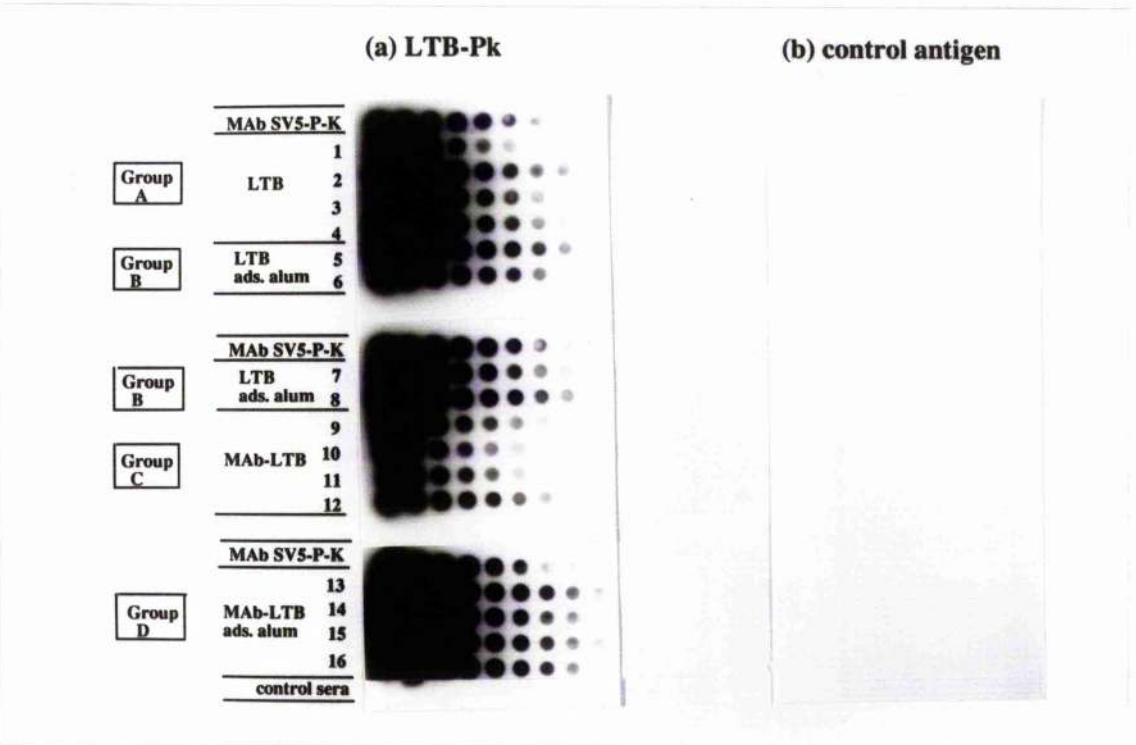


Figure 29. Immunogenicity of LTB-Pk

An example of the ECL-based immune assay used in the detection of antigen-specific antibodies in the sera of immune mice.

Groups of four Balb/c mice were immunized three times with the vaccines shown in Fig.28. Ten days after the last immunization, the sera was assayed for antibodies to LTB-Pk or a control antigen, ovalbumin. The sera was diluted 1:200 and from this starting dilution, serial diluted 1:2 in the wells of a terasaki plate, then incubated with nitrocellulose filters that had been pre-coated with either LTB-Pk or ovalbumin. As a control, sera from non-immunised mice were similarly treated. Bound antibody was detected with an HRP-conjugated anti-mouse immunoglobulin (Ig) antibody, ECL and autoradiography.

Group A: mice immunized with soluble LTB-Pk (LTB); Group B: mice immunized with LTB-Pk adsorobed onto alum (LTB ads. alum); Group C: mice immunized with LTB-Pk linked to MAb SV5-P-k (MAb-LTB) and Group D: mice immunized with MAb-LTB adsorbed onto alum (MAb-LTB ads. alum).

Vaccine		mouse number	1	2
Group A	soluble LTB-Pk	1	12800	400
		2	25600	800
		3	25600	400
		4	25600	400
Group B	LTB-Pk ads. alum	5	12800	800
		6	12800	400
		7	51200	400
		8	25600	400
Group C	soluble MAb-LTB	9	12800	≤100
		10	25600	≤100
		11	12800	≤100
		12	12800	≤100
Group D	MAb-LTB ads. alum	13	51200	≤200
		14	51200	800
		15	51200	800
		16	51200	200

Table 2. Serum titres to LTB-Pk and GST-Pk

The table shows the total serum titres (as detected by HRP-conjugated anti-mouse-Ig antibody) in vaccinated mice to LTB-Pk (column 1) and the Pk-epitope (column 2) following three immunizations with the vaccines described in Fig. 28.

Except for the mice that were immunised with MAb-LTB alone, all mice exhibited some immunity to the Pk-tag. However, the actual antibody titres were consistently small, irrespective of the immunising agent, with an average titre of only 400-800.

Thus, the Pk-tag is a poor systemic immunogen, a finding that supports the observations of Randall and colleagues [1994], and therefore, suggests that the majority of the immune response generated to LTB-Pk is directed at LTB.

SECTION E. Systemic Immunogenicity of Partially Purified p17-MAb-LTB complexes.

E.1. Introduction.

In this section of the results, the preliminary findings on whether the conjugation of recombinant SIV antigens to LTB-Pk via MAb SV5-P-k was a successful strategy for promoting humoral and cell-mediated immunity to the recombinant SIV antigen, following parental administration, are presented.

The Pk-linked SIV antigen chosen for use in the initial immunisation studies was the recombinant gag gene product His-p17-Pk. This recombinant protein had several important attributes that increased its attractiveness as a SIV target antigen. For example, it could be over-expressed in *E.coli* to high levels [Hanke *et al.*, 1994] and was easily purified from the cell lysates by nickel affinity chromatography. Also, incorporation of His-p17-Pk into solid matrix-antibody-antigen complexes had previously been shown to induce recombinant p17-specific humoral and cell-mediated immune responses following intraperitoneal immunisation [Hanke *et al.*, 1994].

E.2. LTB-Pk enhances the immune response to recombinant SIV antigens in vivo.

To investigate whether conjugation of Pk-linked SIV proteins to LTB-Pk via MAb SV5-P-k, led to an enhancement of the immune response to the recombinant SIV

protein, SIV-MAb-LTB immune complexes were constructed and their immunogenicity were examined *in vivo*. p17-MAb complexes were constructed as described in Section C.3 and the purified p17-MAb complexes divided into two equal aliquots. One of the aliquots was then mixed with the appropriate concentration of ammonium sulphate purified LTB-Pk, to create p17-MAb-LTB complexes with a molar ratio of p17-MAb:LTB-Pk of 1:1. The second aliquot of p17-MAb was retained and used in a separate group of immunisations (below).

Groups of four Balb/c mice aged 6-8 weeks were immunised intraperitoneally with the equivalent of 1μg His-p17-Pk alone (Group A) or after adsorption onto alum (Group B), p17-MAb alone (Group C) or with alum (Group D), p17-MAb-LTB alone (Group E) or with alum (Group F) in a final volume of 200μl. Prior to immunisation, 30μl of each vaccine was subjected to 15% (w/v) SDS-PAGE and the protein bands visualised by staining the gel with Coomassie blue to confirm that each vaccine preparation had an identical concentration of the His-p17-Pk and, in the case of the vaccines composed of p17-MAb-LTB, LTB-Pk (Fig.30).

All mice were immunised three times intraperitoneally, on days 0, 14 and 37. Ten days after the second and third immunisations, the mice were bled and the sera examined for antibodies specific for His-p17-Pk, and a control antigen, ovalbumin. Antigen-specific antibodies were again detected by the ECL-immune assay, described previously. An example of the assay is shown in Fig.31, the antibody titres to His-p17-Pk after two and three immunisations with the appropriate vaccines, presented in Table 3, columns 1 & 2, respectively.

With the exception of soluble His-p17-Pk all the vaccines investigated were capable of eliciting anti-His-p17-Pk specific antibodies, the level of immunity increasing with each consecutive immunisation (Table 3, compare columns 1 and 2). None of the mice in Group A responded to His-p17-Pk, demonstrating that, on its own, the purified recombinant protein is not immunogenic. However, when His-p17-Pk was administered in the presence of alum (Group B), the adjuvant enhanced the immune response to the recombinant protein, though the antigen-specific antibody titres obtained varied

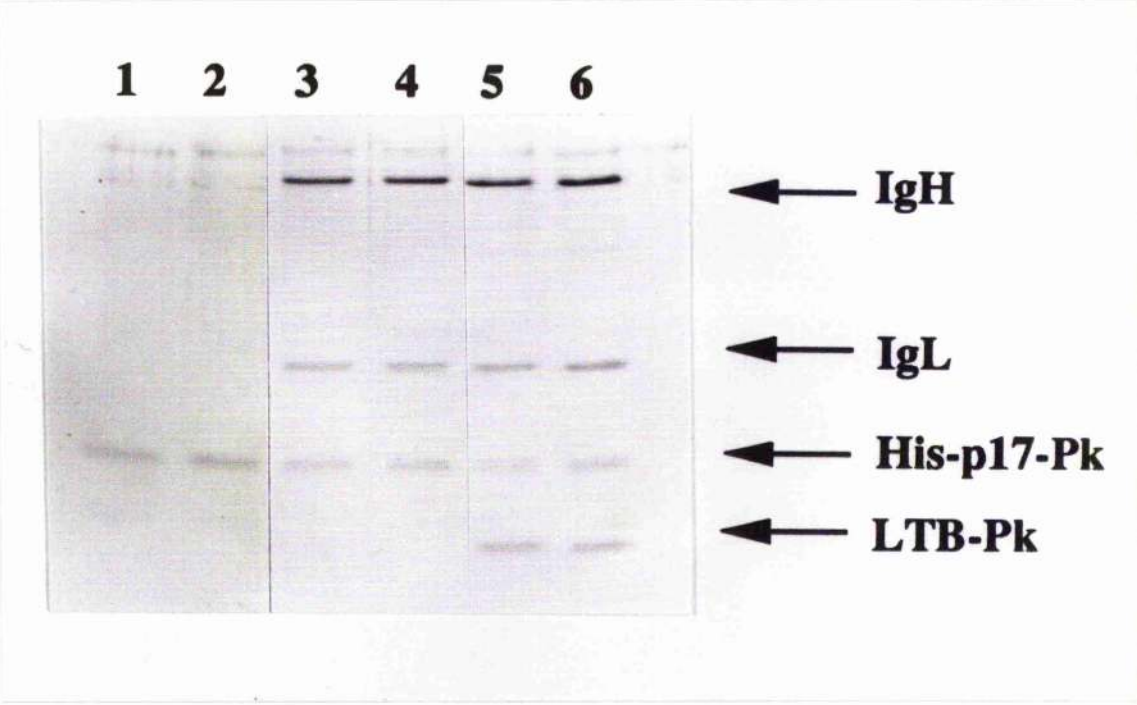


Figure 30. Comparative analysis of His-p17-Pk-based vaccine formulations.

All vaccine formulations were made up to a final volume of 200µl with PBS and 30µl removed for analysis. The samples were electrophorised through a 15% (w/v) SDS-polyacrylamide gel and the protein concentrations estimated by staining the gel with Coomassie blue.

Lane 1: soluble His-p17-Pk; lane 2: His-p17-Pk adsorbed onto alum; lane 3: His-p17-Pk conjugated to MAb SV5-P-k (p17-MAb); lane 4: p17-MAb adsorbed onto alum; lane 5: p17-MAb linked to LTB-Pk (p17-MAb-LTB); lane 6: p17-MAb-LTB adsorbed onto alum.

remarkably within the vaccination group. Similarly, the immunogenicity of His-p17-Pk could be enhanced by presenting the recombinant protein to the immune system as part of an immune complex either alone (Group C) or after adsorption onto alum (Group D). Having said this, it was evident that p17-MAb complexes were far less immunogenic on their own, than when administered with alum, with one mouse in Group C having no detectable serum immunity specific for His-p17-Pk even after three immunisations with p17-MAb.

Examination of the sera from mice vaccinated with p17-MAb-LTB complexes (Group E) for antibodies reactive with His-p17-Pk, revealed immunisation with p17-MAb-LTB complexes alone resulted in an approximately 4-8 fold enhancement in the immune response to His-p17-Pk, in comparison to the immunity induced following vaccination with soluble p17-MAb. Furthermore, the level of immunity detectable was relatively consistent throughout the mice of Group E. Adsorption of p17-MAb-LTB complexes (Group F) onto alum resulted in minor enhancement (2-fold) in the immunogenicity of p17-MAb-LTB complexes.

When the immune sera from each mouse was re-assayed for antibodies specific for LTB-Pk (using the ECL-based immune assay and purified LTB-Pk as the solid-phase target antigen), only the mice in Groups E and F, demonstrated an antibody-specific response to the recombinant enterotoxin subunit. On average, the level of immunity directed at LTB-Pk was 6-8 fold higher than the response to the His-p17-Pk component of the vaccine, this being particularly noticeable when p17-MAb-LTB complexes were administered along with alum (data not shown). This bias of the immune system in favouring the induction of immunity to the LTB moiety of LTB-conjugates, has been demonstrated by others [Schodel *et al.*, 1990]. Examination of the immune sera for antibodies specific for the Pk-epitope, also demonstrated that very little of the immune response was directed at this determinant (data not shown) which supports the previous findings that the Pk-epitope is a poor systemic immunogen.

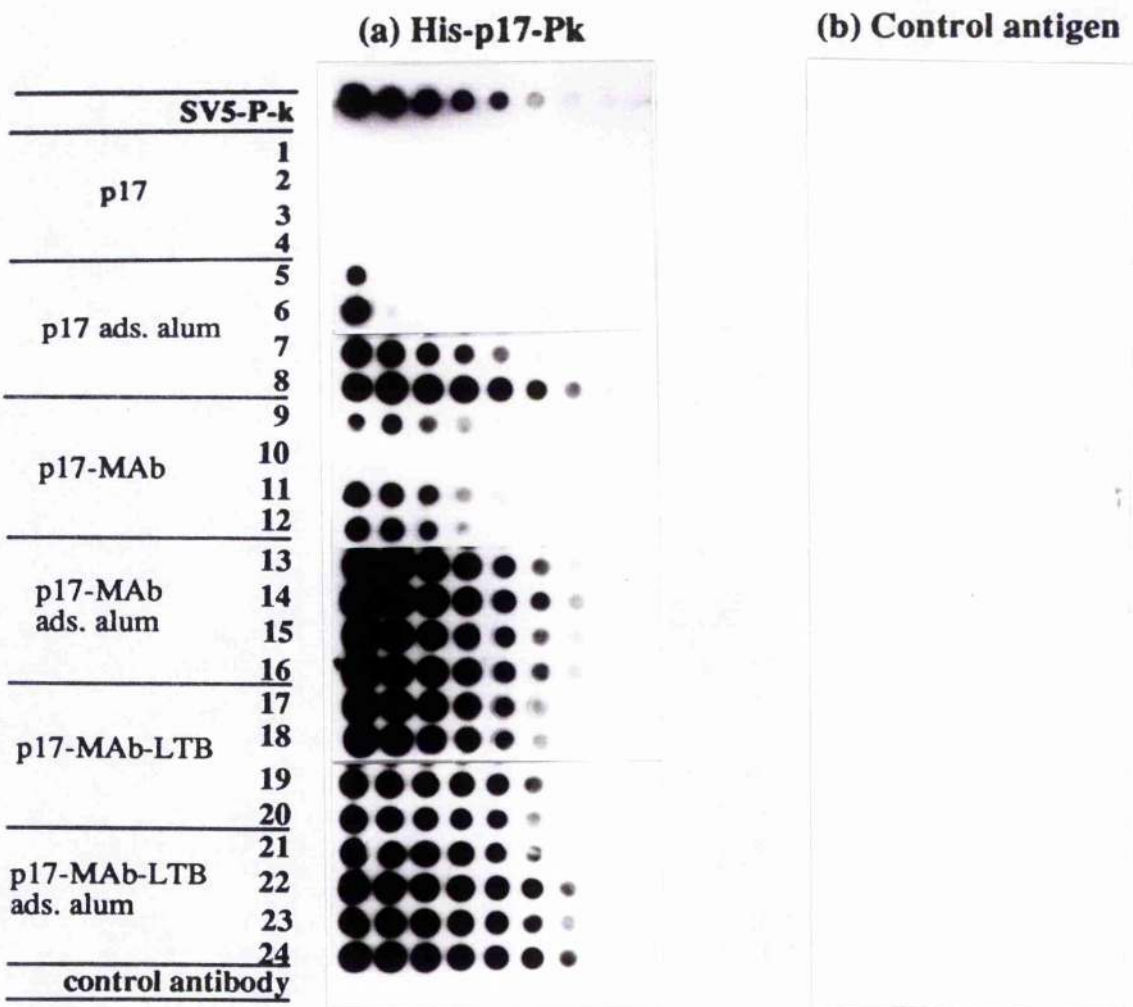


Figure 31. Induction of systemic immunity to His-p17-Pk
Groups of four Balb/c mice were immunized three times with soluble His-p17-k (p17), His-p17-Pk adsorbed onto alum (p17 ads. alum),His-p17-Pk conjugated to MAb SV5-P-k (p17-MAb), p17-MAb adsorbed onto alum(p17-MAb ads. alum), p17-MAb linked to LTB-Pk (p17-MAb-LTB) or p17-MAb-LTB adsorbed onto alum (p17-MAb-LTB ads. alum). All mice were immunized with the equivalent of 1µg of His-p17-Pk for each vaccine formulation. Antibodies specific for His-p17-Pk or a control antigen, ovalbumin, were measured using an ECL-based immune assay. The sera were diluted 1:100 and from this starting dilution, serial diluted 1:2 in the wells of a terasaki plate. The dilutions were then incubated with nitrocellulose filters that had been pre-coated with either purified His-p17-Pk or ovalbumin. Bound antibody was detected with an HRP-conjugated anti-mouse Ig antibody, ECL and autoradiography.

vaccine		mouse number	1	2	3
Group A	soluble p17	1	≤100	≤100	≤100
		2	≤100	≤100	≤100
		3	≤100	≤100	≤100
		4	≤100	≤100	≤100
Group B	p17 ads. alum	5	≤100	200	≤100
		6	200	400	200
		7	400	25600	400
		8	1600	6400	≤100
Group C	p17-MAb	9	200	3200	≤100
		10	200	≤100	≤100
		11	1600	3200	≤100
		12	400	1600	≤100
Group D	p17-MAb ads. alum	13	3200	12800	≤100
		14	6400	25600	≤100
		15	6400	25600	≤100
		16	3200	25600	1600
Group E	p17-MAb-LTB	17	6400	12800	1600
		18	6400	12800	3200
		19	6400	25600	3200
		20	1600	12800	3200
Group F	p17-MAb-LTB ads. alum	21	6400	25600	1600
		22	3200	25600	3200
		23	6400	25600	3200
		24	6400	25600	12800

Table 3. Serum titres to His-p17-Pk in immune mice.
The table shows the total antibody titres (as detected by HRP-conjugated anti-mouse Ig antibody after two (column 1) and three (column 2) immunizations with the vaccines described in Fig.30. The serum response to His-p17-Pk after three immunizations was further qualified by analysing the level of anti-recombinant p17 antibodies that were detectable by HRP-conjugated Protein A (column 3)

Thus, linkage of His-p17-Pk to LTB-Pk by way of a Pk-specific antibody bridge, can help potentiate the immune response to the recombinant SIV protein. Furthermore, the potency of p17-MAb-LTB complexes in the absence of alum, indicate that the presence of LTB-Pk in p17-MAb-LTB complexes may bypass the need for additional adjuvants.

E.3. Characterisation of the antibody response to His-p17-Pk and LTB-Pk.

Different isotypes of antibodies exist, each having its own particular immunological role in host defence. The murine IgG_{2a} isotype is an antibody that is thought to play a crucial role in the protection against viral infection [Coutlier *et al.*, 1987]. It was of interest to investigate whether conjugation of His-p17-Pk to LTB-Pk helped modulate the antibody response to the recombinant SIV protein.

Sera from each mouse in a vaccination group were incubated with His-p17-Pk-coated nitrocellulose sheets and bound antibodies were classified into Protein A-binding [Protein A (Pr.A) preferentially interacts with murine antibodies of the IgG_{2a}, IgG_{2b} and IgG_3 subclasses] and non-Pr. A binding antibodies (usually antibodies of the IgG_1 subclass) by detecting bound antibody with HRP-conjugated Pr. A.

None of the mice from vaccination Groups A-D had anti-His-p17-Pk specific antibodies of an isotype that was recognised by HRP-conjugated Pr. A (Table 3, column 3). Thus, in these experiments, and in contrast to the results of Randall *et al.*, 1994, immunisation with His-p17-Pk or p17-MAb complexes in the presence or absence of alum, did not induce detectable levels of anti-recombinant SIV antibodies of the IgG_{2a}, IgG_{2b} or IgG_3 subclasses. In contrast to this finding, immunisation with p17-MAb-LTB did induce anti-His-p17-Pk and LTB-Pk-specific antibodies of an isotype detectable with the Pr. A conjugate. Similarly, when the sera from vaccinated mice were assayed for Pr.A binding and non-binding antibodies specific for LTB-Pk, the sera from mice vaccinated with p17-MAb-LTB complexes alone or in the presence of alum, contained very high levels of Pr-A binding antibodies (data not shown).

These results suggest that conjugation of His-p17-Pk to LTB-Pk via MAb SV5-P-k can enhance the immune response to the recombinant SIV protein, but also, that LTB-Pk helps modulate the isotype of the antibody response to His-p17-Pk.

E.4. p17-MAb-LTB complexes prime the immune system to SIV.

The previous assays relied on the recognition of the recombinant SIV protein, His-p17-Pk, by antigen-specific sera as an assessment of each vaccines potential to induce immunity to SIV.

To determine whether vaccination with either His-p17-Pk, p17-MAb or p17-MAb-LTB alone or after adsorption onto alum led to serum antibodies that could recognise p17 from an SIV infected cell, the sera within each vaccination group was pooled and reacted in Western blots with viral proteins from SIV-infected cells. Briefly, proteins present in the lysate of an SIV infected cell were separated on a 15% (w/v) SDS-polyacrylamide gel and the denatured proteins electroblotted onto nitrocellulose. The nitrocellulose was cut into 0.5cm x 7cm strips, and individual strips were incubated with a 1:800 dilution of pooled sera from each vaccination group or with control sera from non-immunised mice. Bound antibodies were detected by autoradiography after incubating of the strips with an HRP-conjugated anti-mouse Ig, followed by ECL buffers and autoradiography (Fig. 32).

Using this method, no antibodies could be detected in the sera of control mice (lane 1) or mice immunised with His-p17-Pk (lane 2), or p17-MAb (lane 4), the last finding being rather surprising since immunisation with p17-MAb stimulated an antibody response specific for His-p17-Pk. The sera from mice immunised with His-p17-Pk in the presence of alum (lane 3), reacted only weakly with p17. In contrast, sera from mice immunised either with p17-MAb complexes adsorbed onto alum (lane 5) or p17-MAb-LTB complexes alone (lane 6) or with alum (lane 7), specifically interacted with both p17 and its precusor molecule, p57. It was particularly interesting to note that the immune sera from mice vaccinated with p17-MAb-LTB complexes gave a stronger response to the SIV proteins than immunisation with p17-MAb complexes in the presence or absence of alum,

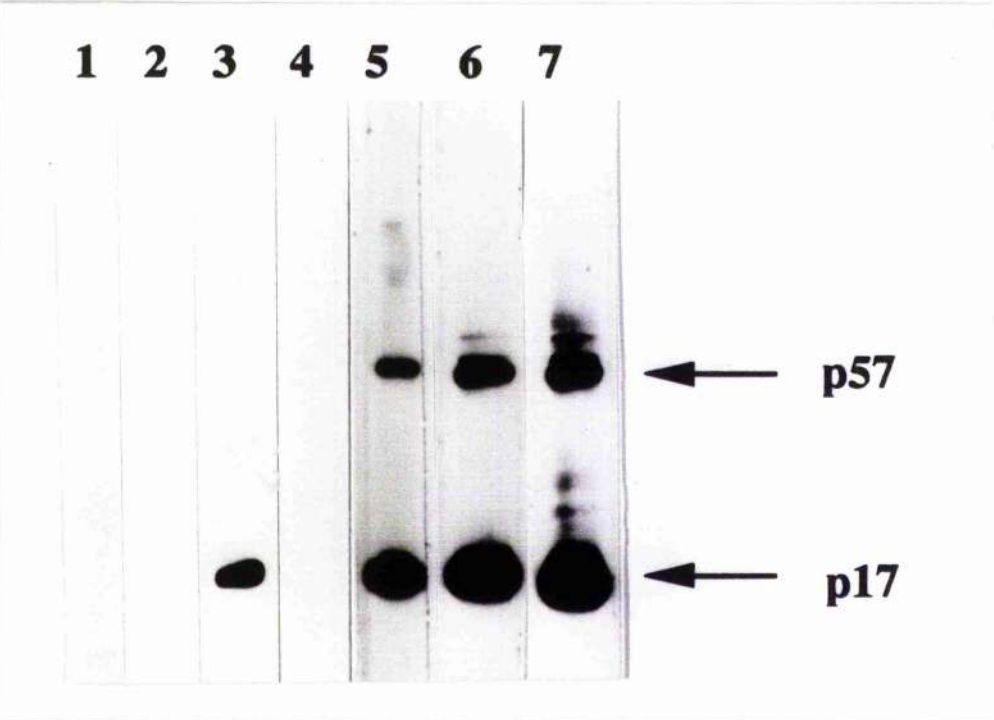


Figure 32. Induction of immunity to p17 from SIV-infected cell lysates.

Viral proteins from a SIV-infected cell lysate were separated through a 15% (w/v) SDS-polyacrylamide gel, then electroblotted onto a nitrocellulose filter and the filter cut into strips. The sera within each vaccination group (see below) was pooled and diluted 1:800, then incubated with the protein-coated nitrocellulose strips. As a control, sera from non-immunized mice was similarly treated. Bound antibodies were detected with an HRP-conjugated anti-mouse Ig antibody, ECL and autoradiography.
Lane 1 represents control sera, lanes 2-7 represent sera from mice immunized three times with p17 (lane 2), p17 ads. alum (lane 3), p17-MAb (lane 4), p17-MAb ads. alum (lane 5), p17-MAb-LTB (lane 6), or p17-MAb-LTB ads. alum (lane 7).

thereby supporting the previous observation that conjugation of p17-MAb complexes to LTB-Pk may bypass the need for additional adjuvants to efficiently prime the immune system.

E.5. Induction cell-mediated immunity to His-p17-Pk by immunisation with p17-MAb-LTB complexes.

Lymphokines secreted from $CD4^+$ T cells, are known to control *in vivo* immunoglobulin isotype selection [Finkelman *et al.*, 1990]. Since all naive B cells normally secrete IgM during primary antigenic stimulation, the presence of IgG antibodies to His-p17-Pk and LTB-Pk following immunisation with p17-MAb-LTB complexes, is an indication of the involvement of cell-mediated immunity in the generation of the antigen-specific B cell response to His-p17-Pk and LTB-Pk.

To investigate whether immunisation with p17-MAb-LTB complexes led to efficient priming of the cell-mediated immune system and the generation of His-p17-Pk and LTB-Pk-specific T cells, the lymphocytes from mice immunised three times intraperitoneally, as described above, were examined in lymphocyte proliferation assays for their ability to respond to specific stimulation with His-p17-Pk or LTB-Pk six months after the final immunisation. For comparison, the lymphocytes from mice that had been immunised with His-p17-Pk or p17-MAb, in the presence or absence of alum, were similarly analysed. The spleens from two mice in each vaccination group, or from non-immunised control mice, were removed and single-cell suspensions prepared. 100µl samples of the unfractionated splenocyte suspension at a concentration of approximately 10^6 cells/ml, were then added to the wells of a 96-well plate, and stimulated *in vitro* for 5 days at 37°C/5% CO_2 with purified His-p17-Pk or ammonium sulphate-purified LTB-Pk at the concentrations shown in the legends of Figs. 33(a) & (b). As positive and negative controls, the cells were also cultured with medium alone or 2µg/ml phytohaemagglutinin (PHA, a well-known T cell mitogen), respectively. During the final 18 hours of the stimulation, 0.5µCi [^{3}H]-thymidine was added to each well in the assay and after harvesting the cells onto glass fibre filters, the proportion of [^{3}H]-thymidine incorporated

into the DNA of proliferating cells was determined in a β-scintillation counting [Fig. 33(a)]. The assay was performed in triplicate and the data is presented as counts per minute (cpm) ± standard error of mean (SEM).

The immune cells from each vaccination group and naive cells showed an enhanced response following mitogenic stimulation with PHA (see legend of Fig.33). Furthermore, there was no evidence the lymphocytes from immunised or control mice proliferated non-specifically when cultured in medium alone (see legend of Fig.33). Upon stimulation with His-p17-Pk, the greatest proliferative response occurred with immune cells recovered from mice immunised intraperitoneally with either p17-MAb-LTB or p17-MAb-LTB adsorbed onto alum, there being little difference in the antigen-specific response between the two vaccination groups.

In contrast to this finding, the immune cells from mice vaccinated with purified His-p17-Pk or p17-MAb complexes showed an enhanced immune response to the recombinant SIV protein, if the purified protein or immune complexes had been adsorbed onto alum prior to administration, though the actual response was evidently lower than the proliferative response demonstrated by the primed lymphocytes from mice vaccinated with p17-MAb-LTB complexes. In all cases, the response to His-p17-Pk was highly specific, since lymphocytes from naive, non-immunised mice failed to proliferate upon stimulation with the recombinant SIV protein.

Enhanced proliferative responses were also evident when primed cells from p17-MAb-LTB immunised mice were challenged i*n vitro* with LTB-Pk [Fig.33 (b)]. Once again, adsorption of the complex onto alum only had minor effects on the proliferative response to LTB-Pk. None of the mice immunised with purified His-p17-Pk or p17-MAb with or without alum, nor naive mice, proliferated in response to stimulation with LTB-Pk. Thus the proliferative response seen for primed cells from p17-MAb-LTB vaccinated mice was highly specific and not due to the ammonium sulphate precipitated LTB-Pk non-specifically stimulating the lymphocytes.

These results suggest that immunisation with p17-MAb-LTB complexes may efficiently prime the cell-mediated immune system to His-p17-Pk and LTB-Pk. In

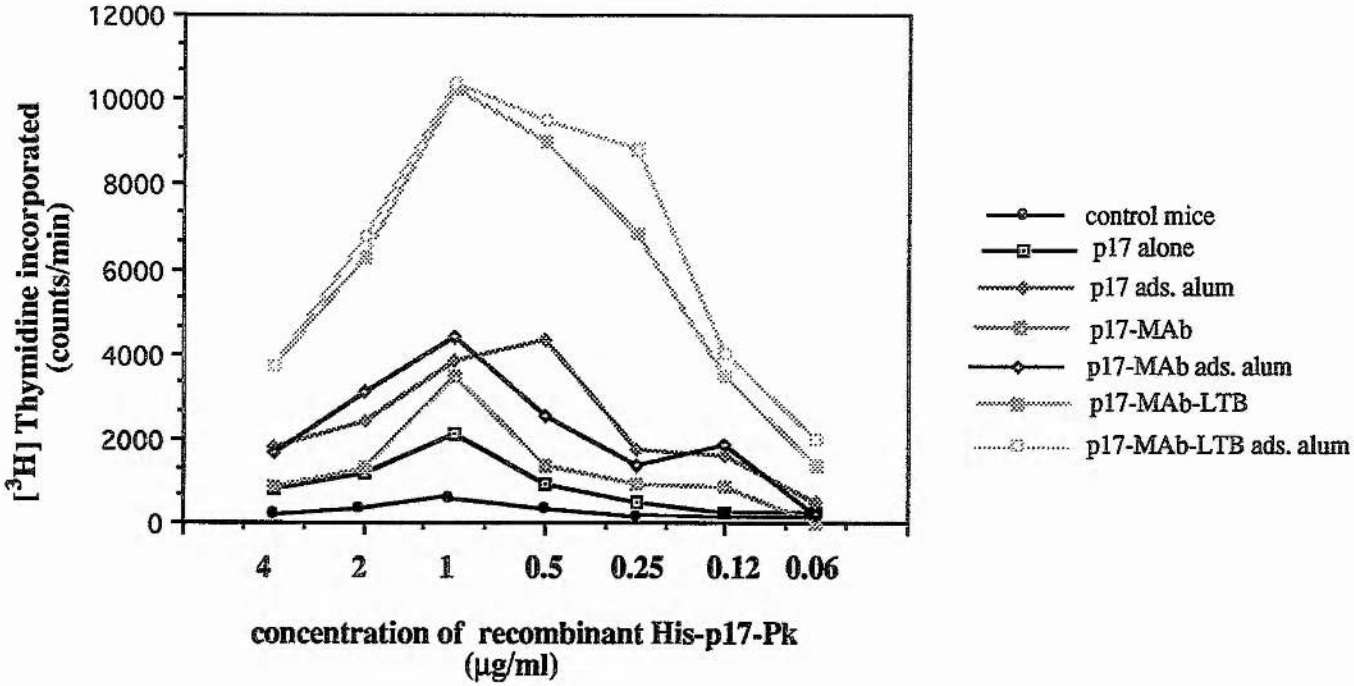


Figure 33(a). Lymphocyte proliferative response to His-p17-Pk

Single cell populations were prepared from the spleens of two mice immunised three times intraperitoneally with the vaccines shown, or non-immunised control mice. The cells were cultured with recombinant p17 at the concentrations indicated above for 5 days. Specific stimulation of the lymphocytes was determined by quantitating the amount of tritiated thymidine incorporated during the last 18 hours of culture.

All background counts (cells cultured in medium alone) were ≤ 800 cpm and have been automatically subtracted. The counts for mitogenic stimulated cultures (cultures stimulated with PHA) were 17,500-20,000. The results are presented as mean±standard deviation for triplicate cultures.

N.B. the standard deviations were so small, they fail to show up on the graph.

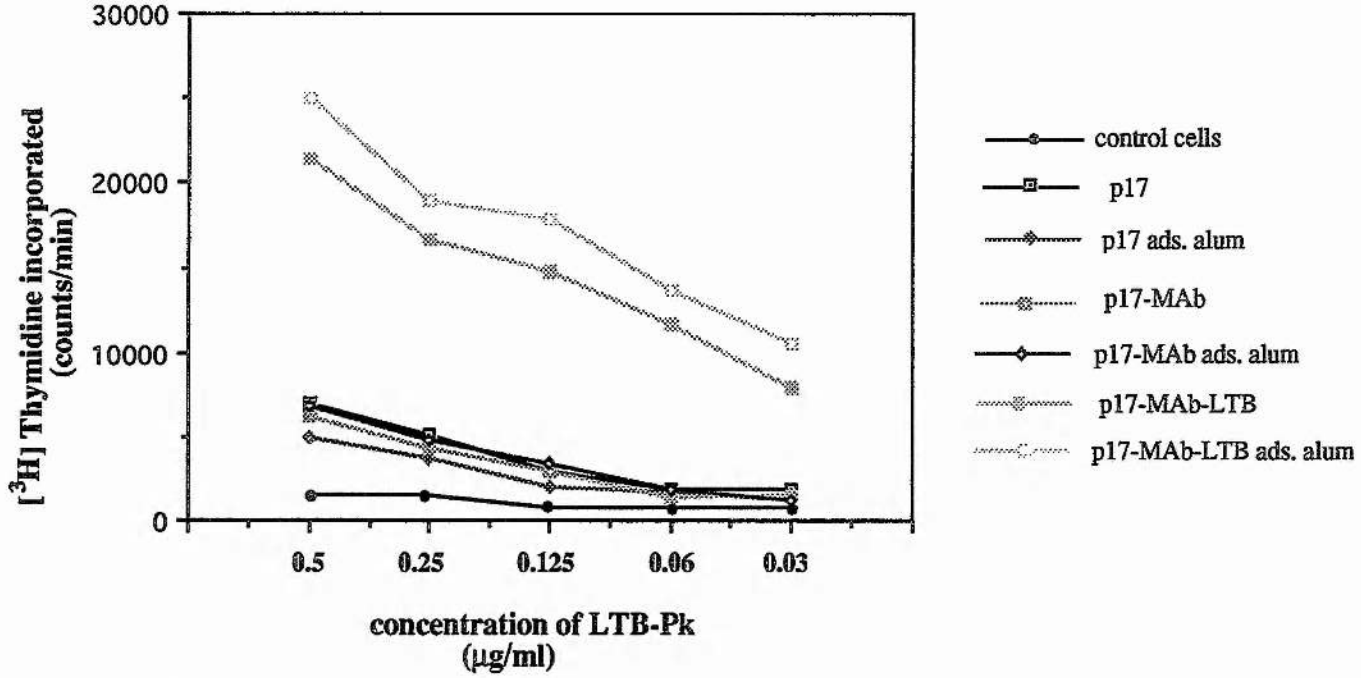


Figure 33 (b). Lymphocyte proliferative response to LTB-Pk

Single cell populations were prepared from the spleens of two mice immunised three times intraperitoneally with the vaccines shown, or non-immunised control mice. The cells were cultured with purified LTB-Pk at the concentrations indicated above for 5 days. Specific stimulation of the lymphocytes was determined by quantitating the amount of tritiated thymidine incorporated during the last 18 hours of culture.

All background counts (cells cultured in medium alone) were ≤ 800 cpm and have been automatically subtracted. The counts for mitogenic stimulated cultures (cultures stimulated with PHA) were 17,500-20,000. The results are presented as mean±standard deviation for triplicate cultures.

N.B. the standard deviations were so small, they fail to show up on the graph.

addition, since the assay was performed six months after the final immunisation, the evidence presented here implies that parental administration of p17-MAb-LTB complexes may lead to the induction of long-term memory cells specific for both His-p17-Pk and LTB-Pk.

E.4. FACscan and cytokine analysis of the proliferating cell type.

Mixed lymphocyte proliferation assays, are normally a reflection on the response of T cells (usually $CD4^+$ T cells) to antigen-specific stimulation. In an attempt to establish whether the lymphocyte population from the unfractionated splenic cultures that responded to stimulation with His-p17-Pk was indeed $CD4^+$ T cells and not B cells, the proliferation assay was repeated and the ratio of cells bearing membrane immunoglobulin or murine CD4 molecules, before and after stimulation, was examined in immunofluoroscence assays.

Single-cell suspensions were prepared from the spleens of vaccinated mice, then added to the wells of a 24-well culture plate in 1ml volumes, at a concentration of approximately 10^7 cells/ml. The cells were then stimulated *in vitro* for five days with an 4μg/ml purified His-p17-Pk at 37°C/5% CO_2. The remaining cells not used in the proliferation assay, were retained to examine the ratio of B cells to $CD4^+$ T cells in the spleens of vaccinated mice prior to antigen-specific stimulation. After a five day culture period, the cells were gently harvested for phenotypic analysis. The ratio of B cells to $CD4^+$ T cells prior to, and after, antigen-specific stimulation was determined by directly staining the unfractionated lymphocyte populations with fluorescein isothiocyanate (FITC)-labelled antibodies specific for murine Igs or CD4 molecules, respectively, and calculating the percentage of fluorescent cells in 10,000 events using the LYSYS program of a Becton Dickson FACscan. An example of the FACscan result is shown in Fig 34. Panels (a) and (b).

In unstimulated spleens, the ratio of B cells to $CD4^+$ T cells in the mixed lymphocyte culture, was approximately 45:40, irrespective of the vaccination group studied, which is consistent with the normal ratio of lymphocytes populating the spleen

[see '*Practical Immunology*'] [Fig. 34, Panel (a)]. However, after stimulation with His-p17-Pk, the proportion of cells detected with FITC-labelled anti-mouse Ig antibody to the proportion detectable with FITC-labelled anti-murine CD4 antibody, was approximately 4:70 [Fig. 34, Panel (b)]. These results suggest that following a five day stimulation with His-p17-Pk, $CD4^+$ T cells predominate.

In a further attempt to characterise the responding cell population in the proliferation assay, the culture supernatant at the end of the stimulation period was analysed for the presence of T cell secreted lymphokines using cytokine-ELISA assays.

Immune cells derived from the spleens of mice vaccinated with p17-MAb complexes in the presence of alum or p17-MAb-LTB complexes alone or after adsorption of the complexes onto alum, were stimulated *in vitro* for five days with medium alone, 2μg/ml PHA or 4μg/ml of His-p17-Pk (as described above), and at the end of the culture period, the supernatant from each culture was examined for IL-2, IL-5 and IFN-γ, using cytokine-ELISA assays (kindly provided by Dr. A. Mowat, Glasgow University). The reason for only examining the cells from these three vaccination groups was due to the fact that the anti-cytokine antibodies used in the assays were both extremely expensive and in limited quantities, thus it was felt that it would be more prudent to only analysis the three vaccination groups that had exhibited the highest proliferative response to His-p17-Pk.

High protein-binding 96-well microtitre plates were pre-coated with anti-IL-2, IL-5 and IFN-γ antibodies for 18 hours at 4^0C. 50μl of the culture supernatants recovered after a five day stimulation period *in vitro*, was added to the first wells in a row and then double-diluted along the row. To measure cytokine levels in unstimulated and mitogen stimulated cultures, the supernatant from lymphocytes stimulated with medium alone or PHA were also included in the assay. After a three-hour incubation at 37^0C, the presence of IL-2, IL-5 and IFN-γ was detected with a biotin-conjugated anti-IL-2, IL-5 and IFN-γ antibodies, followed by extravidin-peroxidase then TMB substrate, and the adsorbance at 630nm measured. The cytokine concentrations in the test supernatants were quantitated by reference to a standard curve constructed using serial dilutions of the standard cytokines.

The level of IL-2 detected in the culture supernatant after a five day specific

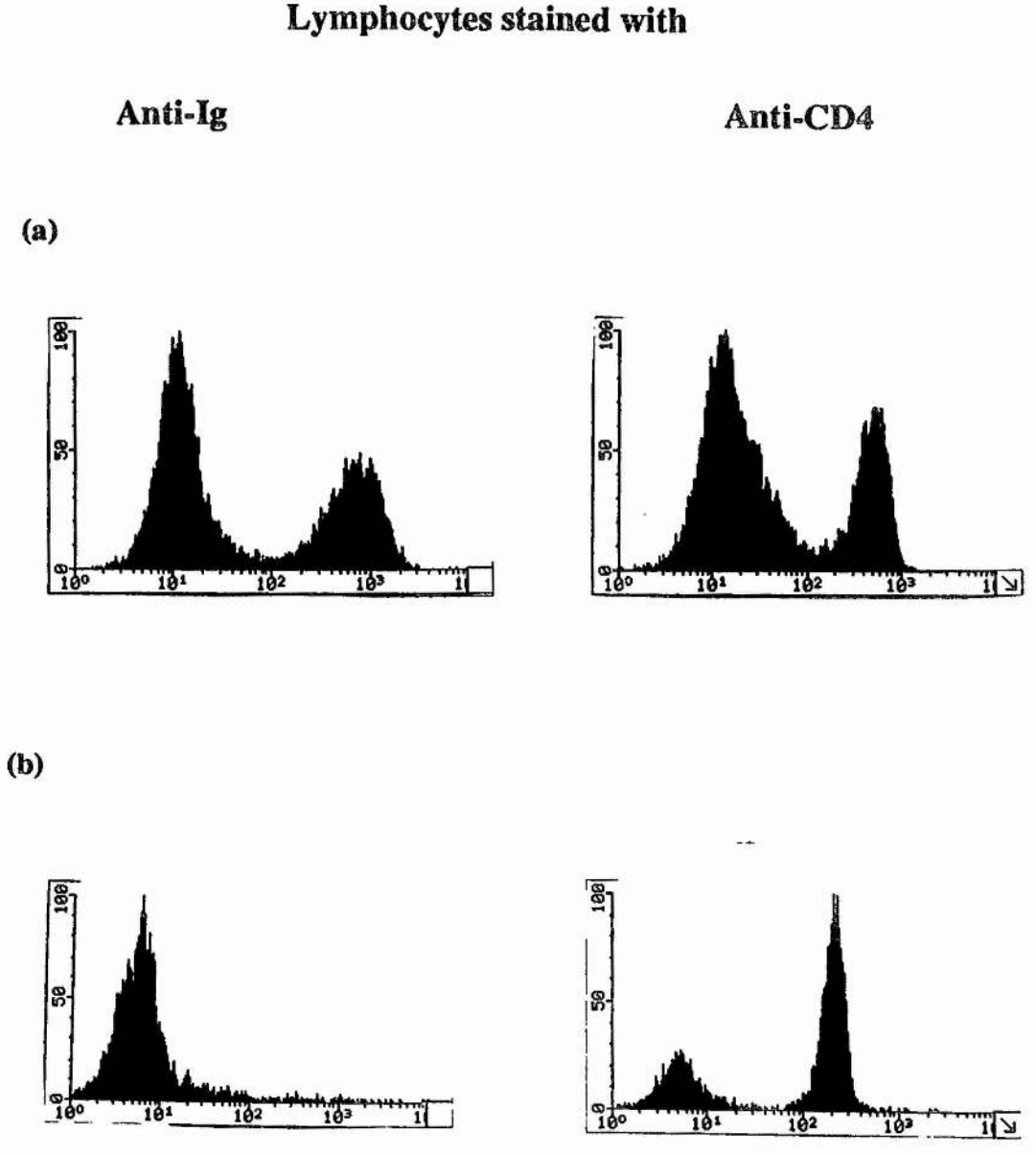


Figure 34. FACscan analysis of His-p17-Pk stimulated cells.
The figure shows an example of the phenotypic analysis of the immune cells from p17-MAb-LTB vaccinated mice, prior to [Panel (a)] and after [Panel (b)] a five day stimulation *in vitro* with His-p17-Pk. The proportion of B cells and $CD4^+$ T cells in an unfractionated population of immune spleen cells was determined by staining the cells with FITC-labelled anti-murine Ig or CD4 antibodies. Positive fluoresence is shown on the right hand side of each graph.

stimulation period was negligible for each vaccination group studied (Table 4) and was well below background levels of IL-2 from unstimulated cultures. Similarly, when the amount of IL-5 was quantitated for each culture, although immune cells from all vaccinated mice were shown to secrete the cytokine, the actual values obtained were small,

Cytokine	1	2	3	4	5
interleukin-2	10 U/ml	110.5 U/ml	2.6 U/ml	1.0 U/ml	0.2 U/ml
interleukin-5	0.3 U/ml	3.2 U/ml	6.9 U/ml	5.4 U/ml	9.2 U/ml
gamma interferon	64 U/ml	116 U/ml	10 U/ml	90 U/ml	143 U/ml

Table 4. Cytokine production by stimulated recombinant p17-primed lymphocytes.
Lymphocytes from mice that had been immunized with p17-MAb ads. alum (column 3), p17-MAb-LTB (column 4) or p17-MAb-LTB ads. alum (column 5) were stimulated *in vitro* for five days with 4µg/ml purified His-p17-Pk . As a control, the cells from each vaccination group were stimualted with medium alone or PHA. At the end of the stimulation period, the culture supernatants were retained and assayed for IL-2, IL-5 and gamma interferon using cytokine-ELISA assays.
N.B. the supernatants from immune cells that had been stimulated with medium alone were pooled (column 1), and used as a control sample to measure background levels of the appropriate cytokine. Similarly, the supernatants from immune cells that had been stimulated with PHA were pooled (column 2) and used as a positive control to ascertain that the cells were capable of producing cytokines following mitogenic activation.

even in PHA stimulated cultures. This minimal detection of IL-5 in the late stages of specific stimulation has been observed by others [Wilson *et al.*, 1991]. Examination of the cultures for IFN-γ proved illuminating. Although antigen-specific stimulation of immune cells from mice vaccinated with p17-MAb complexes in association with alum resulted in detectable IFN-γ levels in the culture supernatant, the actual cytokine level was less than non-specific production of IFN-γ from unstimulated cultures. In contrast, stimulation of lymphocytes from mice vaccinated with p17-MAb-LTB complexes, demonstrated high levels of IFN-γ production, particularly if the complexes were administered in the presence of alum.

These results suggest that the presence of LTB-Pk in the vaccine formulation may lead to the priming of cells that are capable of secreting IFN-γ.

SECTION F. Mucosal Immunogenicity of Partially Purified p17-MAb-LTB complexes.

F.1. Introduction.

The success of soluble p17-MAb-LTB complexes in inducing systemic immunity to His-p17-Pk and viral proteins from SIV infected cells, led to the investigation as to whether such complexes were also capable of stimulating mucosal and/or systemic immunity to the recombinant SIV antigen, following intranasal immunisation. In Section F of the results, the findings of these preliminary mucosal immunisation studies are presented.

F.2. LTB-Pk does not potentiate the immune response to His-p17-Pk following intranasal immunisation.

To determine whether p17-MAb-LTB complexes were immunogenic by the mucosal route, a series of intranasal immunisation studies were conducted. p17-MAb-LTB complexes were constructed as before, and their efficacy compared with purified His-p17-Pk , p17-MAb and His-p17-Pk co-administered with (but not coupled to) LTB-Pk (p17/LTB). This latter

vaccine was included in the studies to determine the relevant importance of the p17-MAb-LTB linkage, as a means of potentiating the response to His-p17-Pk.

Groups of four Balb/c mice, aged 10-12 weeks, were gently anaesthetised and the vaccines, containing the equivalent of 1μg of His-p17-Pk (and where appropriate, 1μg LTB-Pk), were administered intranasally in a final volume of 50μl. All mice were vaccinated three times at two week intervals and the immunogenicity of each vaccine was determined by analysing the sera ten days after the third immunisation for antibodies to His-p17-Pk, LTB-Pk or a control antigen, ovalbumin, using an ECL-based immune assay (Fig.35). The antibody titres to the His-p17-Pk and LTB-Pk are shown in Table 5.

Examination of the sera from non-immune control mice, revealed no detectable serum antibodies specific for any of the test antigens. When the sera from mice immunised with His-p17-Pk (Group A), p17/LTB (Group B), p17-MAb (Group C) or p17-MAb-LTB (Group D) were assayed for antibodies to His-p17-Pk, no serum immunity to His-p17-Pk was detectable. Examination of the sera from each vaccinated mouse for immunity to LTB-Pk, demonstrated that only the mice in Groups B and D contained serum antibodies specific for LTB-Pk, there being little difference in the LTB-Pk-specific antibody titres between the two groups. In addition, none of the mice exhibited detectable antibody responses to the control antigen (data not shown).

To investigate whether the vaccines induced mucosal immunity to His-p17-Pk and/or LTB-Pk, the saliva from each vaccinated mouse was examined for His-p17-Pk- and LTB-Pk-specific IgA antibodies, fourteen days after the third immunisation. As a control, saliva from non-immunised mice were also assayed. Analogous to the findings of the serum analysis, no antibodies specific for His-p17-Pk were detectable in the saliva of vaccinated mice (Fig. 36, Table 6). However, when the saliva was assayed for immunity to LTB-Pk, the mice that were immunised with either p17/LTB or p17-MAb-LTB were shown to contain IgA antibodies-specific for LTB-Pk.

Figure 35 and Table 5. Serum antibody responses to His-p17-Pk and LTB-Pk following intranasal immunization.

Groups of four Balb/c mice were immunized intranasally with His-p17-Pk alone; co-administered with LTB-Pk (P17/LTB); His-p17-Pk conjugated to MAb SV5-P-k (p17-MAb); or p17-MAb linked to LTB-Pk (p17-MAb-LTB), three times. Ten days after the third immunization the sera was assayed for total antibody levels to His-p17-Pk or LTB-Pk , using an ECL-based immune assay. The sera were diluted 1:50 and from this starting dilution, serially diluted 1:2. Bound antibody was detected with an HRP-conjugated anti-mouse immunoglobulin.

N.B. only two mice remained after the third immunization with p17-MAb-LTB, the other two mice died due to an adverse reaction to the anaesthetic.

Figure 35.

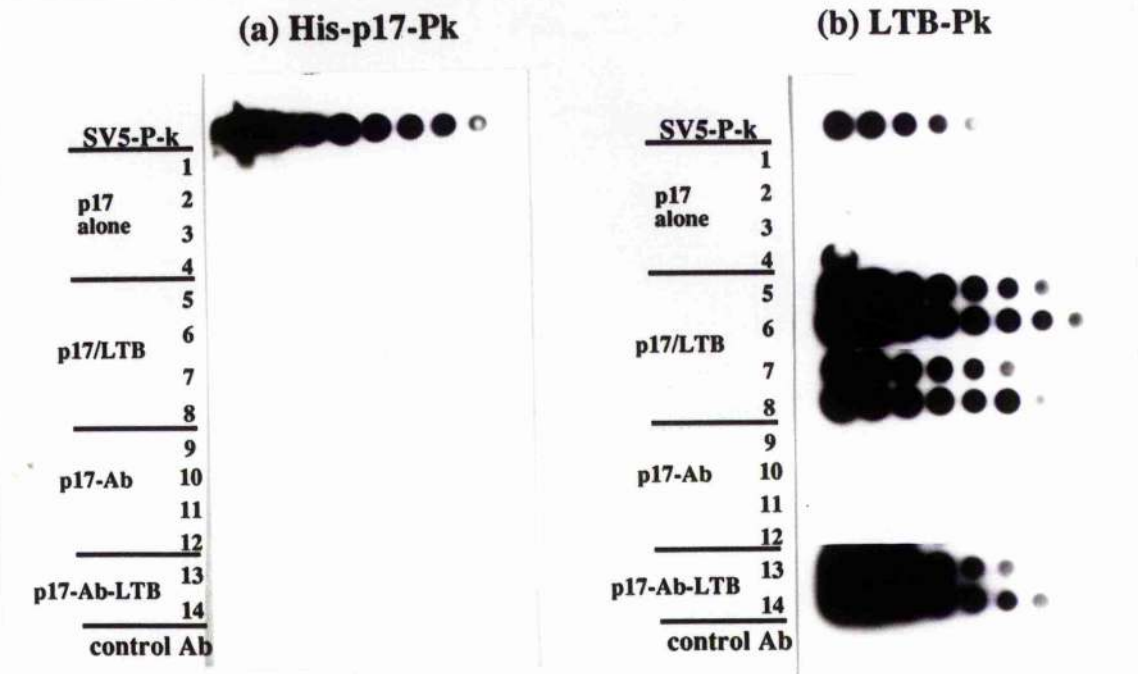


Table 5.

Vaccine		Mouse number	Antibodies to His-p17-Pk	Antibodies to LTB-Pk
Group A	His-p17-Pk	1	≤50	≤50
		2	≤50	≤50
		3	≤50	≤50
		4	≤50	≤50
Group B	p17/LTB	5	≤50	25600
		6	≤50	25600
		7	≤50	12800
		8	≤50	25600
Group C	p17-MAb	9	≤50	≤50
		10	≤50	≤50
		11	≤50	≤50
		12	≤50	≤50
Group D	p17-MAb-LTB	13	≤50	12800
		14	≤50	25600

Figure 36 and Table 6. Antibodies to His-p17-Pk and LTB-Pk in the saliva of intranasally vaccinated mice.

Groups of four mice were immunised intranasally three times with the vaccines shown, and fourteen days after the third immunisation the saliva from immunised mice or non-immunised control mice, were collected and assayed for antibodies to His-p17-Pk, LTB-Pk or a control antigen, ovalbumin, using ECL-based immune assays. The saliva was diluted 1:50 and from this starting dilution, serially diluted 1:2 in the wells of a tersaki plate. The dilutions were then incubated with nitrocellulose filters pre-coated with the appropriate antigen. Bound antibody was detected with a goat anti-mouse IgA antibody, followed by an HRP-conjugated anti-goat Ig, ECL and autoradiography. The antibody titres are tabulated in Table 6. **N.B.** Only two mice remained following three immunisations with p17-MAb-LTB, since two died due to an adverse reaction to the anaesthetic.

Figure 36.

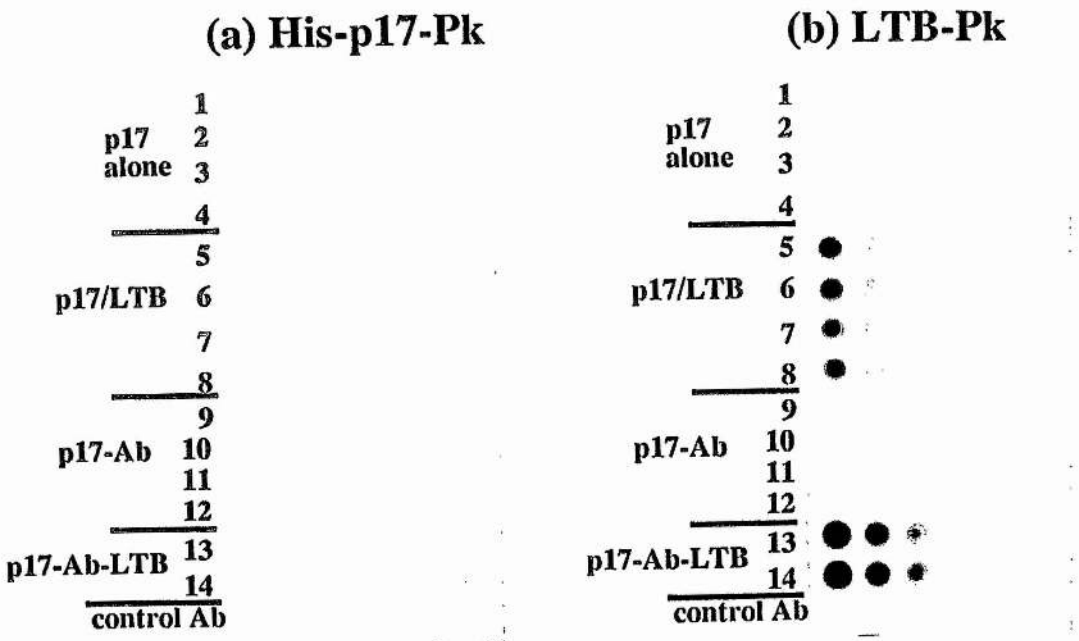


Table 6.

Vaccine		Mouse number	Antibodies to His-p17-Pk	Antibodies to LTB-Pk
Group A	His-p17-Pk	1	≤50	≤50
		2	≤50	≤50
		3	≤50	≤50
		4	≤50	≤50
Group B	p17/LTB	5	≤50	400
		6	≤50	400
		7	≤50	400
		8	≤50	400
Group C	p17-MAb	9	≤50	≤50
		10	≤50	≤50
		11	≤50	≤50
		12	≤50	≤50
Group D	p17-MAb-LTB	13	≤50	1600
		14	≤50	1600

F.3. Instability of His-p17-Pk in saliva.

One possibility for the failure of the vaccines described in Section F.2 to induce immunity to His-p17-Pk following intranasal immunisation, was that His-p17-Pk may undergo rapid degradation upon exposure to the protease-rich mucosal environment. This hypothesis was tested by analysing the stability of His-p17-Pk in saliva, an example of a protease-rich mucosal secretion.

Purified His-p17-Pk at a concentration of 2 or 4μg was mixed with saliva (12% v/v) and the solution was incubated at 37°C. At the times indicated in the legend of Figure, a 15μl sample was removed and mixed with an appropriate volume of 4X disruption buffer. As a control, 2 or 4μg purified His-p17-Pk was incubated with PBS (12% v/v) at 37°C for the full 40 minute incubation period. All samples were electrophoresed through a 15% (w/v) SDS-polyacrylamide gel, and the stability of His-p17-Pk determined by staining the gel with Coomassie blue and comparing the quantity of His-p17-Pk present in control cultures, with the amount remaining after a 10, 20, and 40 minute exposure to saliva (Fig. 37).

A strong band representing His-p17-Pk was detectable in the control cultures that had been incubated for 40 minutes at 37°C with PBS alone (lanes 2 & 6). Thus, at this temperature, His-p17-Pk was not susceptible to breakdown in the absence of protease activity. However, upon exposure to salival proteases, His-p17-Pk was rapidly degraded (lanes 3-5 & 7-9), this being more evident at lower His-p17-Pk concentrations. Therefore it is a strong possibility that the failure of the His-p17-Pk containing vaccines to prime the mucosal and systemic immune systems after intranasal immunisation, was due to rapid degradation by mucosal proteases of the His-p17-Pk component in the experimental vaccines.

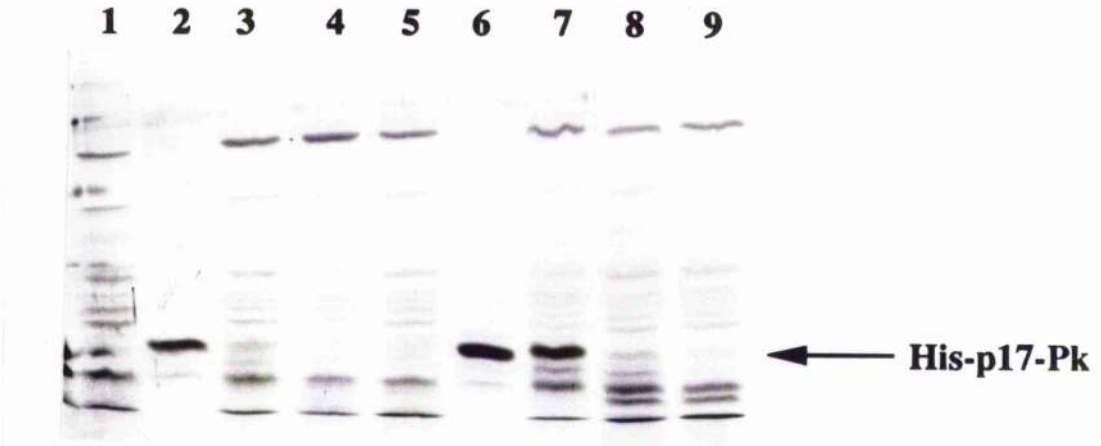


Figure 37. Instability of His-p17-Pk in saliva.
2μg or 4μg of purified His-p17-Pk in 15μl of PBS (lanes 2-5 and 6-9 respectively), were incubated with either 2μl of saliva (lanes 3-5 and 7-9) or 2μl of PBS (lanes 2 & 6) at 37°C for 10 minutes (lanes 3 & 7), 20 minutes (lanes 4 & 8) or 40 minutes (lanes 2, 5, 6 & 9). The polypeptides present in these samples were separated by electrophoresis through a 15% SDS-polyacrylamide gel, then stained with Coomassie blue.
Lane 1 shows an example of the proteins detectable in a 2μl sample of saliva.

SECTION G. Mucosal Immunogenicity of Partially Purified p27-MAb-LTB complexes.

G.1. Introduction.

Section G of the results, describes the finding that His-p27-Pk is more resistant to degradation in saliva, and that p27-MAb-LTB complexes induce systemic and mucosal immunity to His-p27-Pk.

G.2. Resistance of His-p27-Pk to salival degradation.

The susceptibility of His-p17-Pk to degradation by salival proteases led to the search for a suitable recombinant SIV antigen that was more resilient to mucosal degradation. His-p27-Pk, like His-p17-Pk, is a recombinant gag gene product that is expressed in moderately high quantities in *E.coli* (approximately 700µg/litre) and can be purified from cell lysates by nickel affinity chromatography. Thus, it was of interest to establish whether His-p27-Pk could resist rapid destruction by mucosal proteases and therefore, increase the potential of using bivalent immune complexes containing His-p27-Pk on one arm of MAb SV5-P-k and LTB-Pk on the other arm, as mucosal immunogens.

1.5µg of purified His-p27-Pk was mixed with saliva (12% v/v) and incubated at 37°C. 15µl samples of the protein/salival solution were removed after 10, 20, 40, 60 and 120 minutes incubation and mixed with an appropriate volume of 4X disruption buffer. As a control, 1.5µg of His-p27-Pk was mixed with PBS (12% v/v) and incubated for 2 hours at 37°C. Each of the prepared samples were subjected to SDS-PAGE and the polypeptide chains electroblotted onto nitrocellulose. The proportion of His-p27-Pk remaining after each time interval was estimated by an ECL-Western blotting assay, using MAb SV5-P-k as the detection antibody (Fig. 38). The decision to monitor the stability of His-p27-Pk by Western blotting, as opposed to Coomassie blue staining, was due to the fact that the Western blotting assay would also determine if the Pk-epitope was proteolytically cleaved from His-p27-Pk in saliva. Such an outcome would obviously have serious implications on the feasibility of using complexes that rely on the linkage of recombinant Pk-bearing proteins to MAb

SV5-P-k, as mucosal immunogens.

In the absence of salival proteases (lane 1), His-p27-Pk was detected as a single band in the control sample that had been incubated at 37°C. In contrast to this finding, following incubation of His-p27-Pk with saliva, both intact His-p27-Pk and a second protein, presumed to be a degraded product of the recombinant protein, were detectable by MAb SV5-P-k (lanes 3-6). However, it was evident by comparing the strengths of the His-p27-Pk bands after incubation with PBS and saliva, that even after a prolonged two hour incubation with saliva, the majority of His-p27-Pk remained undegraded. These findings suggest that His-p27-Pk may be relatively resistant to mucosal proteolysis.

To determine whether salival proteases would impair the structural integrity of p27-MAb-LTB complexes, the stability of p27-MAb-LTB complexes (prepared in a similar fashion to that described for p17-MAb-LTB complexes) following incubation with saliva, was investigated in immune precipitation assays. The equivalent of 1.5μg of p27-MAb-LTB complexes was incubated with saliva (12% v/v) at 37°C for two hours. As a control, 1.5μg of p27-MAb-LTB complexes were mixed with PBS (12% v/v) and similarly incubated. The stability of the p27-MAb-LTB complexes following incubation with PBS or saliva, was determined by precipitating the complexes onto *St. aureus* and examining the precipitates for the presence of both His-p27-Pk and LTB-Pk, by Western blotting. As controls, His-p27-Pk and LTB-Pk were incubated with *St.aureus* alone, to determine whether the recombinant proteins could precipitate onto *St.aureus* in the absence of MAb SV5-P-k.

As shown in figure 39, neither His-p27-Pk nor LTB-Pk bound to *St. aureus* in the absence of MAb SV5-P-k (lanes 1&2, respectively). However, two bands representing the recombinant proteins were present in the precipitate from the control culture, demonstrating that p27-MAb-LTB complexes were not degraded following a two hour incubation in PBS at 37°C (lane 3). When the stability of p27-MAb-LTB complexes that had been incubated with saliva were examined by Western blotting, it was evident that the whole complex remained intact and could be captured by *St.aureus* (lane 4).

Hence, p27-MAb-LTB complexes were resistant to degradation by salival proteases under the conditions described.

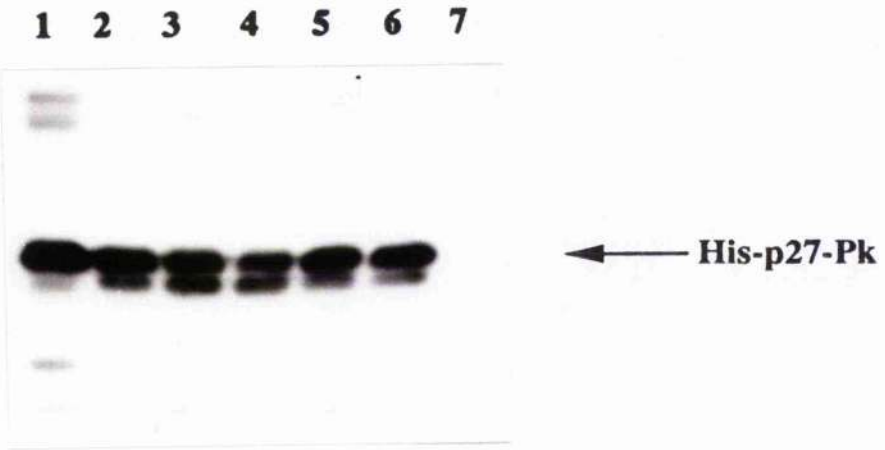


Figure 38. Stability of His-p27-Pk in saliva

The stability of His-p27-Pk after exposure to salival proteases was investigated in a Western blot assay. 1.5µg of His-p27-Pk in 15µl of PBS was incubated with either 2µl of PBS (lane 1) or 2µl of saliva (lanes 2-6) at 37°C for 10 minutes (lane 2), 20 minutes (lane 3), 40 minutes (lane 4), 60 minutes (lane 5) or 120 minutes (lanes 1 & 6). Samples were removed after the appropriate times and the proteins electroblotted onto nitrocellulose. The presence of His-p27-Pk was detected by probing the blot with MAb SV5-P-k followed by HRP-conjugated Protein A, ECL and autoradiography.
Lane 7 represents a control sample of 2µl saliva that had been incubated at 37°C for 120 minutes.

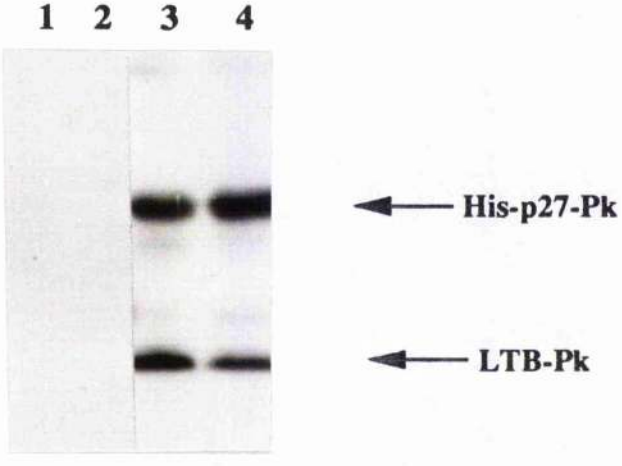


Figure 39. Stability of p27-MAb-LTB complexes after exposure to salival proteases.

The stability of p27-MAb-LTB complexes was investigated in immune-precipitation assays. 1.5µg of p27-MAb-LTB complexes in 15µl of PBS were incubated with either 2µl of PBS (lane 3) or 2µl of saliva (lane 4) for 2 hours at 37°C. The complexes were then mixed with *St. aureus* and the resulting precipitates analysed by Western blot assay for the presence of His-p27-Pk and LTB-Pk. As a control, His-p27-Pk (lane 1) and LTB-Pk (lane 2) were incubated with *St.aureus* , to determine whether the recombinant antigens were bound by *St. aureus* in the absence of the anti-Pk MAb.

G.3. p27-MAb-LTB complexes can induce mucosal and systemic immunity to the recombinant SIV protein, following intranasal inoculation.

On the basis of the evidence outlined in Section G.2., it was decided to investigate whether p27-MAb-LTB complexes were immunogenic following intranasal inoculation.

Eight Balb/c mice, aged 10-12 weeks, were gently anaesthetised with di-ethyl ether and the equivalent of 1.5µg of His-p27-Pk in p27-MAb-LTB complexes was administered into the nasal cavity, in a final volume of 75µl . All mice received two further immunisations of 1µg of p27-MAb-LTB in a final volume of 50µl at two week intervals. Ten days after the third immunization, the serum was analysed by ECL-based immune assays for immunity to His-p27-Pk-, LTB-Pk, GST-Pk or a control antigen, ovalbumin (Fig.40). The antibody titres to the respective antigens are shown in Table 7.

All mice receiving intranasal doses of p27-MAb-LTB complexes contained circulating antibodies reactive with His-p27-Pk, although the actual antibody titres varied between immunised mice [Fig.40, Panel (a) & Table 7, column 1]. High levels of serum antibodies specific for LTB-Pk were also detectable throughout the vaccination group, but in contrast to the variable immunity to His-p27-Pk, the antibody titres to LTB-Pk were relatively consistent [Fig. 40 Panel (b) & Table 7, column 2]. Very little of the immunity generated to His-p27-Pk and LTB-Pk was directed at the Pk-epitope [Fig. 40 Panel (c) & Table 7, column 3], and none of the mice contained antibodies to the control antigen [Fig. 40 Panel (d)]. The sera from immunised mice were also tested for their ability to react with viral proteins in SIV-infected cell lysates, as described in the legend of Fig. 41. As a control, sera from non-immunised mice was also included in the assay.

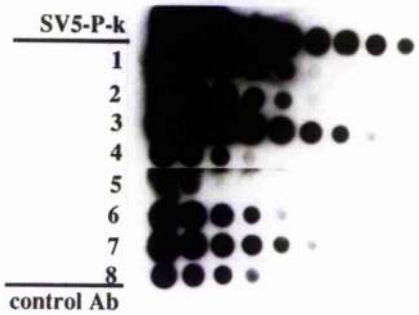
Examination of the resulting autoradiograph revealed that immune sera (lane 1), but not control sera (lane 2), reacted strongly with both p27 and its percursor molecule, p57.

Figure 40. Induction of serum immunity to His-p27-Pk and LTB-Pk by intranasal administration of p27-MAb-LTB complexes.

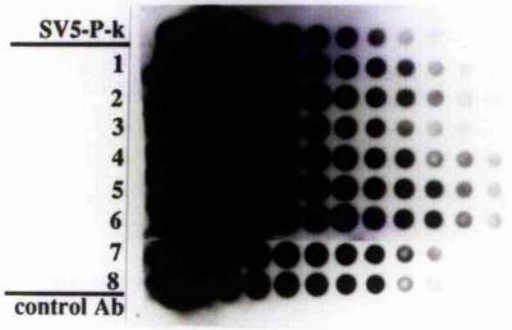
Eight Balb/c mice were immunized three times, intranasally, with the equivalent of 1μg of His-p27-Pk in p27-MAb-LTB complexes. Ten days after the third immunization, the sera was assayed for antibodies to either His-p27-Pk, LTB-Pk, the Pk-epitope (GST-Pk) or a control antigen, ovalbumin, in ECL-based immune assays. The sera was diluted 1:20 and from this starting dilution, serially diluted 1:3 in the wells of terasaki plates, then incubated with individual nitrocellulolose sheets which had been pre-coated with the appropriate antigen. MAb SV5-P-k and sera from non-immunized mice were included in the assay as positive and negative controls respectively. Bound antibody was detected with an HRP-conjugated anti-mouse Ig, ECL and autoradiography.

The figure shows an example of the resulting autoradiograph, the antibody titres are tabulated in Table 7.

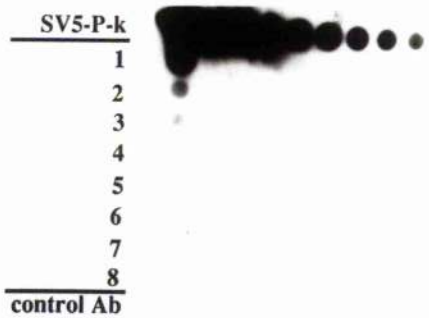
(a) His-p27-Pk



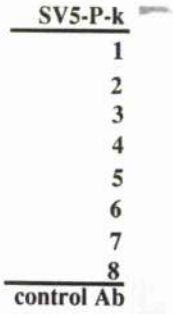
(b) LTB-Pk



(c) GST-Pk



(d) Control antigen



Mouse number	Antibodies in the sera to		
	His-p27-Pk	LTB-Pk	GST-Pk
1	14580	≥3542940	60
2	540	≥3542940	60
3	1620	≥3542940	≤20
4	1620	≥3542940	≤20
5	393660	≥3542940	≤20
6	4860	≥3542940	≤20
7	540	≥3542940	≤20
8	43740	≥3542940	≤20

Table 7. Antibody titres in the sera of mice intranasally immunized with p27-MAb-LTB complexes.
Table shows the total antibody titres to His-p27-Pk, LTB-Pk and GST-Pk in the sera of mice Balb/c mice following three intranasal inoculations with the equivalent of 1μg of His-p27-Pk in p27-MAb-LTB complexes. The sera was assayed 10 days after the third immunization (see Figure 40).

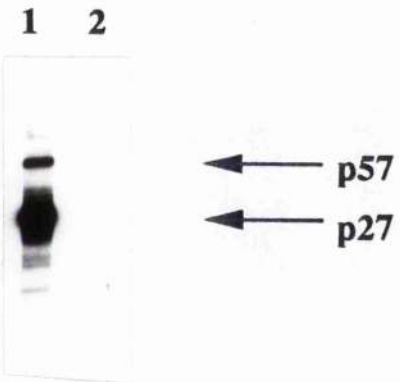


Figure 41. Induction of systemic immunity to SIV following intranasal immunization with p27-MAb-LTB complexes.
The pooled sera from both mice immunized with p27-MAb-LTB complexes (lane 1) and non-immunized mice (lane 2) were incubated with nitrocellulose strips that had been pre-coated with viral proteins from an SIV-infected cell lysate. Bound antibodies were detected with an HRP-conjugated anti-mouse Ig, ECL and autoradiography.

G.4. Analysis of the mucosal response to His-p27-Pk and LTB-Pk in vaccinated mice.

The presence of serum antibodies to p27 and LTB-Pk following intranasal administration of p27-MAb-LTB complexes suggests that the immune complexes were processed at the local mucosal level in the upper respiratory tract. To establish whether the mucosal system was also primed to His-p27-Pk and LTB-Pk following intranasal vaccination with p27-MAb-LTB complexes, both the upper and lower respiratory tract were examined for evidence of an immune response to the recombinant antigens.

(1) Measurement of secretory antibody.

To measure the level of specific-humoral immunity to His-p27-Pk and LTB-Pk in the upper respiratory tract, saliva was collected from pilocarpine treated mice, 11 days after the third intranasal immunisation. Saliva was serially diluted 1:2 from a starting dilution of 1:5 in the wells of a terasaki plate and then incubated with either His-p27-Pk LTB-Pk, or GST-Pk coated nitrocellulose sheets. As a control, saliva from non-immunised mice were similarly treated. Antigen-specific antibodies were visualised upon autoradiography after incubating the sheets with goat anti-mouse IgA followed by an HRP-conjugated anti-goat Ig, exposure to ECL buffers and autoradiography (Fig. 42). The antibody titres to His-p27-Pk and LTB-Pk are shown in Table 8.

Following three intranasal immunisations with p27-MAb-LTB, all vaccinated mice contained anti-LTB-Pk antibodies in the saliva 11 days after immunisation (Column 1), the actual titres varying from 20-320. In contrast to this finding, only minor levels of His-p27-Pk-specific antibodies were detectable in the saliva of two of the vaccinated mice (column 2). When the saliva was assayed for antibodies specific for the Pk-epitope, or a control antigen, no antigen-specific antibodies were detectable (data not shown).

(2) ELISPOT Analysis.

To further analyse the efficacy of intranasal administration of p27-MAb-LTB complexes to induce mucosal immunity to His-p27-Pk, the lungs from vaccinated mice were investigated for immunity to the recombinant antigens using an enzyme-linked immunospot (ELISPOT) assay. Such assays are particularly suitable for investigating

Figure 42 and Table 8. Antibodies in the saliva of p27-MAb-LTB intranasally vaccinated mice specific for His-p27-Pk or LTB-Pk.

Eight Balbl/c mice were immunised intranasally at two week intervals with the equivalent of 1μg of His-p27-Pk in p27-MAb-LTB complexes. Eleven days after the third immunisation, the saliva was collected and assessed for His-p27-Pk and LTB-Pk-specific IgA antibodies as described in the legend of Fig. 40. The antibody titres are given in Table 8.
Please note that in this instance, the saliva was diluted 1:5 and from this starting dilution, serially diluted 1:2.

Figure 42.

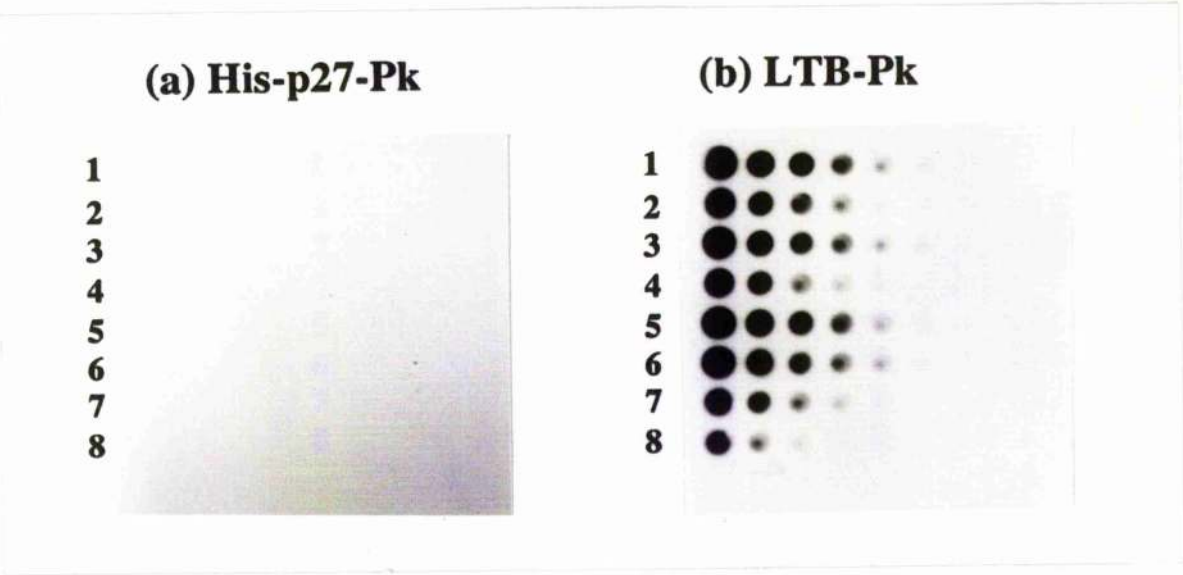


Table 8.

Mouse number	Antibodies in Saliva to	
	His-p27-Pk	LTB-Pk
1	≤5	160
2	≤5	80
3	≤5	160
4	20	80
5	≤5	320
6	20	160
7	≤5	80
8	≤5	20

humoral immunity in tissues that are normally difficult to analyse e.g. Peyer's patches of the gut [Czerkinsky *et al.*, 1983] , and since they measure specific antibody secretion at the single cell level, they are ultimately more sensitive than conventional ELISA's that measure specific antibodies in external secretions. Furthermore, with selective use of isotypically different detection antibodies in the assays, it is possible to monitor the isotype pattern of the antigen-specific antibody secreting cells.

Fourteen days after the third immunisation two mice were killed and their lungs extracted. To measure the difference in the level of anti-recombinant p27 antibodies secreting cells between peripheral and mucosal tissues following intranasal immunisation with p27-MAb-LTB complexes, the spleens were also removed for analysis. For the lungs, the outer epithelial layer was removed by treatment with collagenase A and DNase I, and the cells liberated by straining the digested tissue through nylon mesh. Spleen cells were prepared as described in Methods. As controls, the lungs and spleens from non-immunised, aged-matched Balb/c mice were similarly treated.

The cells were resuspended in ISCOVE's medium containing 10% FCS to give a concentration of approximately 10^6 cells/ml. 100µl of the cell suspension was added to the top wells of His-p27-Pk-coated 96-well microplates and four serial 1:4 dilutions in ISCOVE's medium were performed. The plates were incubated at 37°C, 5% CO_2 in a humidified incubator for 18 hours to allow antibody secretion to occur. After the removal of cells, a 1:1000 dilution of sheep anti-mouse IgM, IgG or IgA antibodies were added to the appropriate wells of replicate plates, followed by a 1:1000 dilution of an alkaline phosphatase goat anti-sheep streptavidin-conjugated antibody. Secreted and bound antibody was visualised as discrete spots, by the addition of alkaline phosphatase substrate to each well, and when the spots were fully developed, the wells were washed gently in distilled water to remove excess substrate. The number of antibody secreting cells (ASCs) of the particular isotype were determined by enumerating the number of spots present under a dissecting microscope (Table. 9).

His-p27-Pk-specific antibodies of all the major isotypes could be detected in the lungs of mice vaccinated three times intranasally with p27-MAb-LTB. Intriguingly, the

Tissue	Antibody Isotype	Number of spot forming cells (sfc) / 10^5 cells secreting antibody specific for His-p27-Pk in Non-immunized mice	Immunized mice
Lungs	IgM	1 ± 1	48 ± 1.5
	IgG	0 ± 0	161 ± 4.0
	IgA	0 ± 0	92 ± 2.8
Spleen	IgM	4 ± 1.5	10 ± 2.0
	IgG	0 ± 0	25 ± 1.5
	IgA	0 ± 0	2 ± 2.0

Table 9. Number of SFC secreting antibodies specific for His-p27-Pk

Pooled lymphocytes from two mice immunized three times intranasally with 1µg of p27-MAb-LTB complexes, or from non-immunized control mice were cultured in 200µl volumes at 10^6 cells /ml in the wells of a microtitre plate that had been previously coated with purified His-p27-Pk. Single cells capable of secreting anti-recombinant p27 antibodies were detected and characterised with anti-mouse isotype specific sera, followed by alakaline phosphatase-conjugated antibody. The level of humoral immunity in the lungs and spleens were estimated by counting the number of spots formed after the addition of a BCIP-based substrate, each spot representing a single anti-recombinant p27 antibody secreting cell.
The results are presented as the mean of duplicate cultures + standard deviation.

predominant anti-His-p27-Pk antibody response was of the IgG isotype, though IgA antibodies specific for the recombinant SIV protein were also present in high numbers. In comparison, although the spleen of vaccinated mice also contained B cells actively secreting antibodies to His-p27-Pk, very few cells secreted antibodies of the IgA isotype, instead IgG secreting cells predominated.

Thus, intranasal administration of p27-MAb-LTB complexes can induce immunity to His-p27-Pk in the lungs of vaccinated mice.

G.5. Cell-mediated immune response.

Recent reports have suggested that, in contrast to popular belief, the mucosal immune system does exhibit memory [Vajdy and Lycke *et al.*, 1993]. To further investigate the efficacy of p27-MAb-LTB complexes as immunogens, the ability of the complexes to induce memory to His-p27-Pk and LTB-Pk was investigated.

Firstly, the spleens from intranasally vaccinated mice were recovered three months after the third intranasal vaccination and analysed for their ability to respond to specific antigenic challenge with purified His-p27-Pk and LTB-Pk in lymphocyte proliferation assays. As a control, the cells from non-immunised, aged-matched mice were similarly analysed. 10^6 cells/ml were either added to the wells of a 96-well microtitre plate (100µl per well), or to the wells of a 24-well microtitre plate (1ml per well). All cells were then stimulated *in vitro* with medium alone, 2µg/ml PHA, purified His-p27-Pk or purified LTB-Pk (see Fig. legend for the concentrations of the recombinant antigens). The plates were incubated at 37°C, 5%CO_2 for five days in a humidified incubator and 0.5µCi of [^{3}H]-thymidine was added to each well of the 96-well microtitre plate for the last 18 hours of the stimulation. The cells in the 96-well plate were then harvested and the amount of [^{3}H]-thymidine incorporated into the DNA of proliferating cells quantitated by a scintillation counting (Fig. 43). The cells in the 24-well culture plate, were gently harvested after a five day stimulation period with 2µg/ml PHA, 4µg/ml purified His-p27-Pk or 0.5µg/ml LTB-Pk and retained for phenotypic analysis, as described in previously.

Although all cells responded to mitogenic stimulation with PHA (see legend of Fig.43), neither immune cells or control cells responded with non-specific proliferation when cultured in medium alone (see legend of Fig.43). There was also no evidence that naive cells from non-immunised mice responded to stimulation with either His-p27-Pk or LTB-Pk. In contrast, a significant enhancement in the proliferative responses to the recombinant proteins was seen when immune cells from mice vaccinated intranasally three months previously with p27-MAb-LTB complexes, with maximum proliferation occurring after stimulation with 4μg/ml purified His-p27-Pk and 0.5μg/ml LTB-Pk.

To determine whether that the cells responding to His-p27-Pk and LTB-Pk were antigen-specific memory T cells, the immune cells in the 24-well plate that had been stimulated with 4μg/ml His-p27-Pk or 0.5μg/ml LTB-Pk were harvested and divided into three equal aliquots of 10^7 cells. One of the aliquots was then incubated with a 1:100 dilution of FITC-labelled anti-mouse Ig, the second aliquot with FITC-labelled anti-murine CD4 and the third aliquot with FITC-labelled anti-murine CD8. As a comparison, the cells that had been stimulated with PHA were similar labelled.

For immune lymphocytes stimulated with the PHA, only the $CD8^+$ T cells and $CD4^+$ T cells were identified as responding to the T cell mitogen (Fig.44). Following a five day stimulation with 4μg/ml of purified His-p27-Pk, both $CD4^+$ T cells and $CD8^+$ T cells were detectable in the stimulated cultures. In contrast, only $CD4^+$ T cells were detectable after stimulation with LTB-Pk, suggesting that intranasal administration of LTB-Pk specifically primes the $CD4^+$ T cell subset. For none of the antigen or mitogen stimulated cultures were B cells detectable in the assay after a five day stimulation period.

Secondly, two mice were given a fourth intranasal boost with 1.5μg of His-p27-Pk in p27-MAb-LTB complexes, three months after the third immunisation, and the sera was assayed for immunity to His-p27-Pk, LTB-Pk, GST-Pk or a control antigen. In both instances, the mice were seen to respond with antibody titres to His-p27-Pk and LTB-Pk similar in magnitude to that seen following three intranasal immunisations (data not shown). This response was highly specific since no antibodies were detectable to either the Pk-epitope or the control antigen.

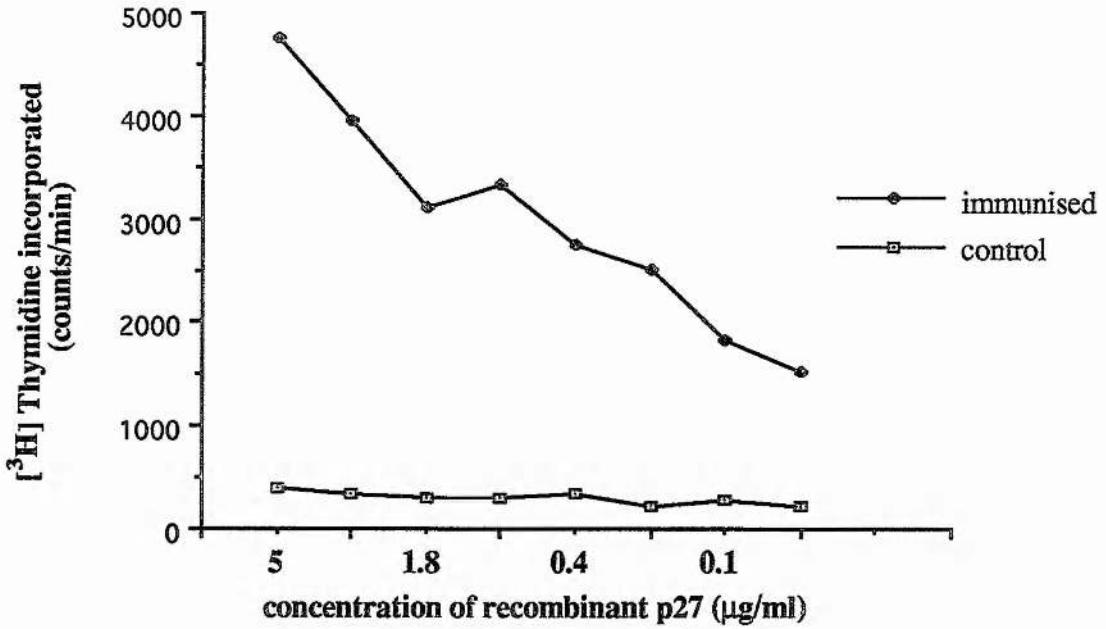


Figure 43(a) Lymphocyte proliferative response to His-p27-Pk

Single cell populations were prepared from the spleens of two mice immunised three times intranasally with p27-MAb-LTB, or non-immunised control mice. The cells were cultured with recombinant p27 at the concentrations indicated for 5 days. Specific stimulation of the lymphocytes was determined by quantitating the amount of tritiated thymidine incorporated during the last 18 hours of culture.
All background counts (cells cultured in medium alone) were ≤ 720 cpm and have been automatically subtracted. All cpm counts for PHA mitogenically stimulated cultures were 15-20,000. The results are presented as mean± standard deviation for triplicate cultures.
N.B. the standard deviations were so small they fail to show up on the graph.

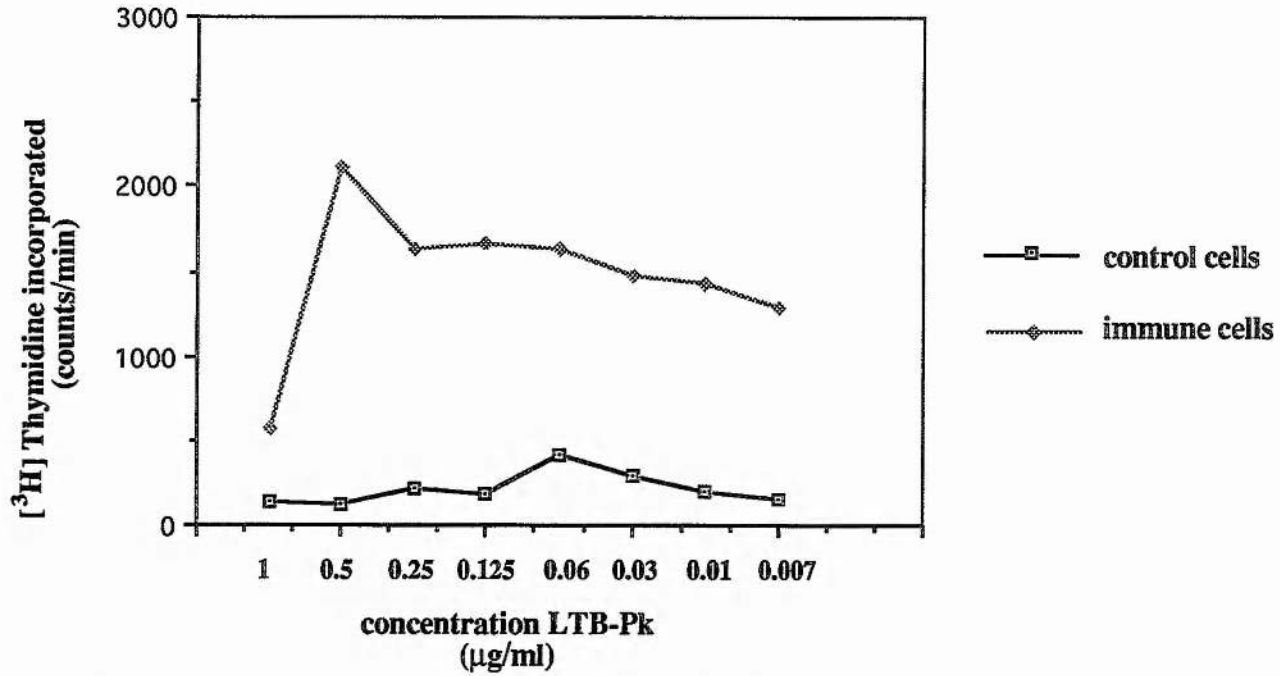


Figure 43(b). Lymphocyte proliferative response to LTB-Pk

Single cell populations were prepared from the spleens of two mice immunised three times intranasally with p27-MAb-LTB, or non-immunised control mice. The cells were cultured with purified LTB-Pk at the concentrations indicated for 5 days. Specific stimulation of the lymphocytes was determined by quantitating the amount of tritiated thymidine incorporated during the last 18 hours of culture.
All background counts (cells cultured in medium alone) were ≤ 720 cpm and have been automatically subtracted. All mitogenic stimulated cultures gave responses of approximately 15-20,000 cpm. The results are presented as mean ± standard deviation for triplicate cultures.
N.B. the standard deviations were so small that they do not appear in the graph.

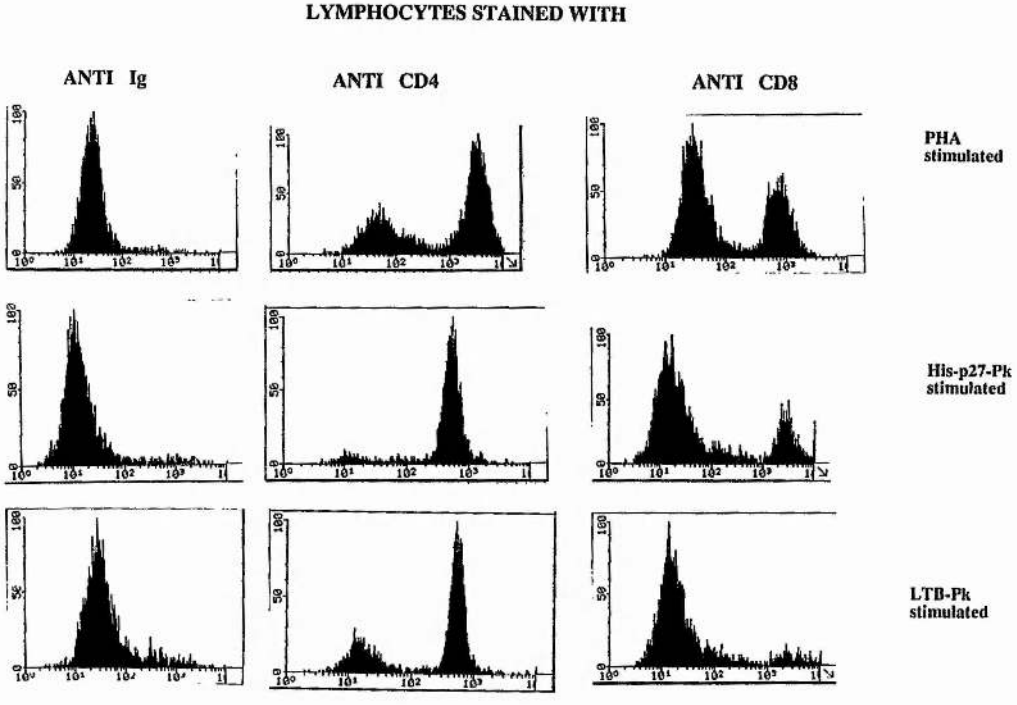


Figure 44. FACscan analysis of recombinant PHA, p27 and LTB-Pk stimulated immune cells.
Immune cells from mice that had been intranasally immunised three times with p27-MAb-LTB complexes were stimulated *in vitro* with either PHA, His-p27-Pk or LTB-Pk for 5 days. The cells were recovered and the proportion of B cells, $CD4^+$T cells and $CD8^+$ T cells present after stimulation with the respective antigens, was measured by FACscan using FITC-labelled anti-murine Ig, CD4 or CD8 antibodies as the detection antibodies.

Thus, these results suggest that intranasal administration of p27-MAb-LTB complexes can lead to anti-recombinant p27 and LTB-Pk memory B and T cells.

SECTION H. Systemic Immunogenicity of Highly Purified p27-MAb-LTB Complexes.

H.1. Introduction.

The development of an efficient purification protocol for LTB-Pk meant, towards the end of this work, that immunogenicity studies using highly purified SIV-MAb-LTB complexes could be performed. In this last section of the results, the initial findings on the ability of such purified complexes to induce humoral immunity to His-p27-Pk following parental immunisation are presented.

H.2. Purified p27-MAb-LTB can prime the systemic immune system to SIV.

To determine the efficacy of purified SIV-MAb-LTB complexes as systemic immunogens, p27-MAb-LTB complexes were constructed as described previously but incorporating LTB-Pk that had been purified by ion-exchange chromatography. Groups of four Balb/c mice were immunised intraperitoneally with the equivalent of 1μg purified His-p27-Pk (Group A), purified His-p27-Pk co-administered with 1μg purified LTB-Pk (Group B), p27-MAb (Group C) or p27-MAb-LTB (Group D). Each mouse was immunised three times on days 0, 14 and 37 and 10 days after the second and third immunisations the sera was analysed for the total level of antibodies to His-p27-Pk, LTB-Pk, GST-Pk or a control antigen, ovalbumin, using the ECL-based immune assay. As before, prior to immunisation, a 30μl sample of each vaccine was removed and after electrophoresis through a 15% SDS-polyacrylamide gel, similiar concentrations of His-p27-Pk in each vaccine were verified by staining the gel with Coomassie blue (data not shown). Figure 45 shows the ECL-based result of antibody levels to His-p27-Pk and the

control antigen after two and three immunisations, respectively. The full antibody titres to His-p27-Pk, LTB-Pk, and GST-Pk are presented in Table 10.

Examination of the sera for anti-His-p27-Pk antibodies revealed a significant difference in the immune response to the recombinant SIV protein between the different groups of mice. In contrast to the findings with purified His-p17-Pk in Section E.2, purified His-p27-Pk was immunogenic when administered alone, with maximum levels of immunity obtainable after three inoculations, though there was some evidence that complexing the recombinant protein to MAb SV5-P-k resulted in a decrease in the immunogenicity of His-p27-Pk (Table 10, columns 1 &2).

Intriguingly, immunisation with p27-MAb-LTB complexes resulted in an average 8-10-fold enhancement in the immune response to the recombinant SIV protein, compared to vaccination with purified His-p27-Pk co-administered with LTB-Pk. However, after three immunisations there was no significant difference in the antibody titres obtained from immunising with p27/LTB, and p27-MAb-LTB. These results indicate that, although the overall level of humoral immunity to His-p27-Pk is similar in mice immunised with His-p27-Pk co-administered with LTB-Pk and mice immunised with p27-MAb-LTB complexes, p27-MAb-LTB complexes are more efficient at priming the immune system since fewer vaccinations are needed for the complex to induce appreciable antibody titres to the recombinant SIV protein. Sera from immunised mice also reacted with p27 (and its precursor p57) present in the total lysate of SIV infected cells, the strength of the signals obtained suggesting that p27-MAb-LTB complexes were more efficient at stimulating anti-p27 immunity (Fig. 46).

Examination of the sera response to LTB-Pk revealed only the LTB-Pk vaccine constructs induced antibodies to the recombinant enterotoxin subunit (data not shown), and there was little difference in the antibody titres obtained following immunisation with p27/LTB and p27-MAb-LTB. When the sera was re-assayed against GST-Pk to determine the proportion of antibodies that were interacting with the Pk epitope, only vaccination with p27/LTB seemed to induce a response to the tag antigen (Table 10, column 4).

Figures 45 (a) and (b). Antibody response to His-p27-Pk after immunisation with purified p27-MAb-LTB complexes.
Groups of four mice were immunised with the 1μg of purified His-p27-Pk alone (p27), co-administered with 1μg purified LTB-Pk (p27/LTB), His-p27-Pk conjugated to MAb SV5-P-k (p27-MAb) or p27-MAb linked to LTB-Pk (p27-MAb-LTB). All mice were immunised three times and the sera assayed ten days after the second [Fig.45 (a)] and third [Fig.45 (b)] immunisations. For sera taken after the second immunisation, the sera was diluted 1:20 and from this starting dilution, serially diluted 1:2. For sera assayed after the third immunisation, the starting dilution was 1:100, then the sera was once again serially diluted 1:2. All sera were assayed against His-p27-Pk and a control antigen, ovalbumin using the ECL-based immune assay. Bound antibody was detected with an HRP-conjugated anti mouse Ig, ECL and autoradiography.

Figure 45 (a)

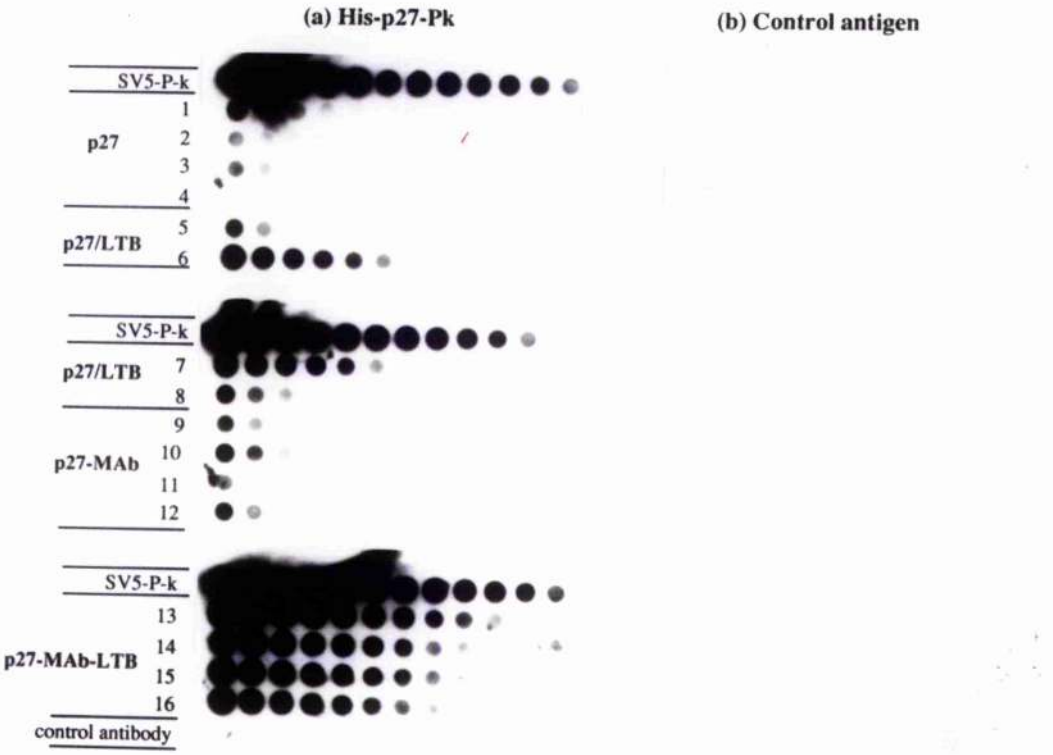
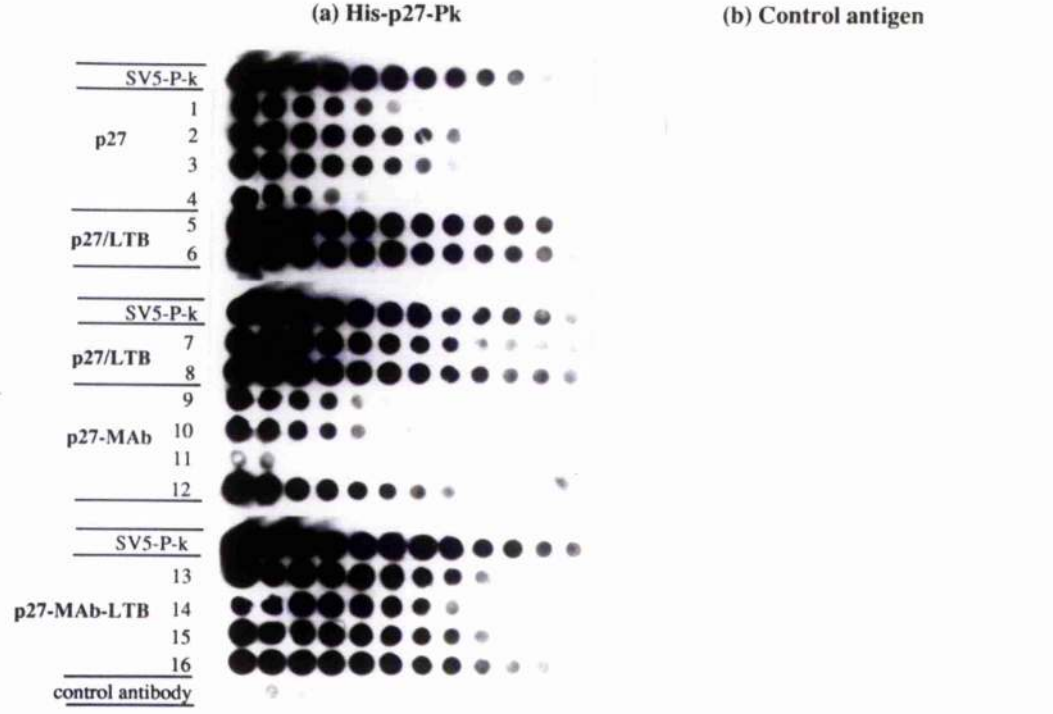


Figure 45 (b)



vaccine		mouse number	1	2	3	4
Group A	p27	1	160	6400	200	200
		2	160	51200	≤100	3200
		3	160	51200	100	800
		4	60	6400	≤100	≤100
Group B	p27/LTB	5	320	≥204800	400	3200
		6	2560	≥204800	800	6400
		7	1280	≥204800	≤100	6400
		8	320	≥204800	≤100	12800
Group C	p27-MAb	9	160	6400	≤100	200
		10	160	12800	≤100	100
		11	80	800	≤100	≤100
		12	160	102400	≤100	≤100
Group D	p27-MAb-LTB	13	10240	≥204800	800	≤100
		14	10240	≥204800	800	800
		15	10240	≥204800	3200	≤100
		16	5120	≥204800	800	800

Table 10. Antibody titres to His-p27-Pk following intraperitoneal immunization with purified recombinant p27-based vaccine formulations.

The table shows the total serum titres to His-p27-Pk in mice immunized with the vaccines shown following two (column 1) and three (column 2) immunizations. The serum response following three immunizations with the appropriate vaccine was further characterized by analysing the level of anti-recombinant p27 antibodies that were detectable with HRP-conjugated Protein A (column 3) and the total antibody titres to the Pk-epitope (column 4).

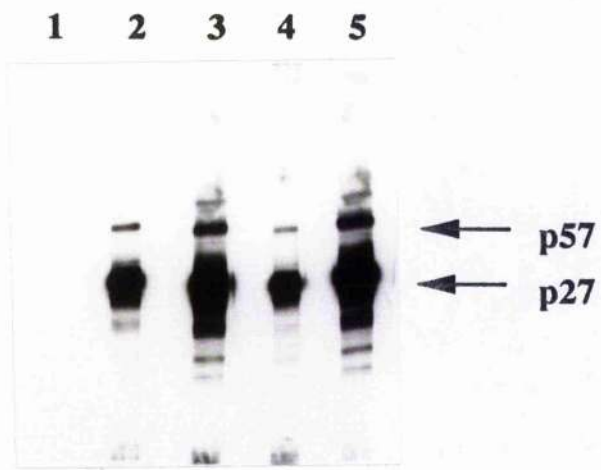


Figure 46. Detection of antibodies to SIV p27 and p57
The sera within each vaccination group (see below) or control sera from non-immunized mice, were pooled, diluted 1:400 and then reacted with nitrocellulose strips that had benn pre-coated with viral proteins from an SIV-infected cell lysate. Bound antibodies were detected with an HRP-conjugated anti-mouse Ig antibody, ECL and autoradiography.
Lane 1 represents control sera, lanes 2-5 represent sera from mice imunized three times with purified: p27 (lane 2); p27/LTB (lane 3); p27-MAb (lane 4) or p27-MAb-LTB (lane 5).

DISCUSSION

1. Expression of LTB-Pk.

A number of plasmid vectors have been constructed that permit the insertion of DNA sequences at the C-terminus of the LTB gene [Sandkvist *et al.*, 1987]. One such vector is pTRH101R, which encodes full length LTB with a short amino acid extension, containing five unique restriction sites, at the carboxyl terminus. A double stranded oligonucleotide sequence, encoding a 14-amino acid tag (Pk) was successfully inserted between the Hind III and Spe I restriction sites, and the novel plasmid pTRH-Pk encoding LTB-Pk, was mobilised into both *Escherichia coli*, and *Vibrio sp.*60. Studies on the expression of LTB-Pk in either organism demonstrated that the recombinant enterotoxin subunit was under control of the inducible *tac* promoter, a hybrid *trp-lac* promoter [Bagadassarian *et al.*, 1983] and as such, LTB-Pk could only be expressed following induction with IPTG. One striking observation resulting from these expression studies was that LTB-Pk resided in two different cellular locations, depending on the host bacterium. In *E.coli*, LTB-Pk was detectable by Western blot assays in the cellular lysates but not the extracellular fluid, suggesting LTB-Pk remained cell-associated in this organism. However, in *Vibrio sp.*60, although minor levels of LTB-Pk were present in the cellular fraction, the vast majority of LTB-Pk was secreted into the external milieu. Previous studies on the cellular location of LTB following expression in *E.coli* and *Vibrio* species has demonstrated a similar pathway of secretion to that of LTB-Pk, suggesting that the addition of the Pk-tag does not interfere with the normal translocation of LTB subunits. Further, since LTB-Pk was detected in Western blotting assays using anti-Pk MAb SV5-P-k as the detection antibody, this implies that; (1) the Pk-epitope is not removed prior to, or during export of LTB-Pk and, (2) the Pk-sequence is not structurally modified following expression in *E.coli* or *Vibrio sp.*60 since the epitope retains its antigenicity when coupled to the C-terminus of LTB.

Studies into the efficacy of *E.coli* and *Vibrio sp.*60 as expression systems for LTB-Pk, investigated the ideal conditions that promote maximal yields of LTB-Pk . In *E.coli*, maximal levels of LTB-Pk production occurred when the culture conditions were

changed from 37°C to 30°C. However, the high level expression of LTB-Pk at this reduced temperature only occurred following induction with IPTG concentrations of 1-0.5mM and there was an indication that LTB-Pk was inefficiently processed under these conditions; the detection of a second protein in the assay that had a slightly higher molecular weight than the LTB-Pk monomers, suggested that the N-terminal signal sequence present on precursor molecules of LTB (and thus LTB-Pk) [Dallas and Falkow, 1980], was not efficiently cleaved to yield the mature protein. Enhanced expression of LTB-Pk in *Vibrio sp.* 60 could also be achieved by decreasing the temperature during induction, yet in contrast to *E.coli*, the level of LTB-Pk synthesis was consistent throughout an IPTG dose range of 1-0.05mM. In addition, there was no evidence that LTB-Pk was susceptible to degradation following expression in *Vibrio sp.*60. These findings clearly demonstrate that *Vibrio sp.*60 is a more efficient expression system for LTB-Pk than *E.coli*, both in terms of stability of LTB-Pk and economy-wise, since reduced amounts of IPTG are necessary for maximal synthesis. Further support for the suitability of *Vibrio sp.*60 as an expression system for LTB-Pk, was provided by the evidence demonstrating that LTB-Pk was expressed at high levels in the *Vibrio sp.*60. Quantitative GM1-ELISA assays estimated that the amount of LTB-Pk detectable in the culture medium from IPTG-induced *Vibrio sp.*60 reached levels of 6 mg/litre after a 6 hour induction, increasing to 12 mg/litre if the induction period was permitted to continue overnight. *Vibrio* species have previously been investigated as expression systems for LTB-fusions [Amin and Hirst, 1994; Schodel *et al.*, 1991]. However in those studies, there was a limitation to the length of time the induction could be performed, due to on-going proteolysis of the fused epitope. Such a finding was not demonstrable for LTB-Pk. This was first suggested when the level of LTB-Pk with anti-Pk reactivity in the extracellular medium was shown to markedly increase in correlation with increasing induction times and subsequently confirmed in immune-precipitation assays, where all inducible protein was found to bind to *Staphylococcus aureus* in the presence, but not absence, of MAb SV5-P-k .

These results suggest that not only is *Vibrio sp.*60 a convenient expression system for LTB-Pk, but also the high level of expression and remarkable stability of LTB-Pk

increases the recombinant enterotoxin subunit's attractiveness as a component of novel vaccines.

2. Characterisation of LTB-Pk.

Characterisation studies on LTB-Pk demonstrated that if LTB-Pk molecules were not exposed to heat-treatment prior to SDS-PAGE, LTB-Pk migrated with an apparent molecular weight of 45kD. However, when LTB-Pk was boiled prior to SDS-PAGE, the recombinant enterotoxin had a greater electrophoretic mobility, migrating with an apparent molecular weight of approximately 13.5 kD. These differential migration patterns showed that LTB-Pk is an heat-labile oligomer, similar to native LTB [Hardy *et al.*, 1988] and demonstrate that the presence of the Pk-addition does not interfere with subunit-subunit association. This observation was confirmed when heat-treated and untreated LTB-Pk were analysed by Western blot, where it was shown that MAb SV5-P-k could detect monomeric and oligomeric structures, respectively.

Further characterisation studies examined whether the presence of the Pk epitope influenced the ganglioside binding properties of LTB-Pk, the natural substrate for native LTB [Finkelstein and Clements, 1979]. Such studies demonstrated that LTB-Pk could efficiently bind to the glycolipid, GM1-ganglioside. These findings not only show that the Pk tag did not prevent binding of LTB to its glycolipid substrate, but also indicated that following binding, the interaction of the Pk-specific antiserum with the Pk-epitope of LTB-Pk was not inhibited. Thus the orientation of LTB-Pk once bound to GM1-ganglioside is such that the Pk-epitope is readily available for subsequent association with MAb SV5-P-k, and increases the chances that MAb SV5-P-k containing immune-complexes can be targeted to GM1-ganglioside through the affinity of LTB-Pk for the glycolipid. This was subsequently shown in GM1-ELISA assays, where SIV-MAb-LTB complexes could bind to the glycolipid, but SIV-MAb complexes could not.

It is interesting that these characterisation studies have demonstrated that a 14-amino acid sequence addition does not apparently interfere with the physiological properties of native LTB. Sandkvist and colleagues [1987] have shown that alterations to the carboxy

terminus of LTB can severely inhibit both the pentamerisation and glycolipid-binding properties of LTB. The exact sequence requirements involved in this inhibition are unknown.

3. Concentration of LTB-Pk.

Purification of recombinant antigens for subsequent use in vaccine formulations is highly important since, contaminating material may increase the toxicity of the vaccine as well as induce adverse immunological reactions, e.g. hypersensitivity responses. The secretion of LTB-Pk from *Vibrio sp.*60 is clearly an attractive attribute, since it means that there are fewer contaminants from which LTB-Pk has to be purified. This latter point is supported by SDS-PAGE analysis of *Vibrio sp.*60 culture supernatants, where LTB-Pk and another protein, of unknown function, are the major protein species detectable. However, the secretion of LTB-Pk entails that the protein has to be purified from large volumes of medium, thus necessitating that a preliminary concentration step be employed prior to the purification procedures. Early attempts to concentrate LTB-Pk from culture supernatants employed the traditional approach of ammonium sulphate precipitation. The minimum concentration of ammonium sulphate necessary to efficiently precipitate LTB-Pk was determined empirically, by observing the proportion of LTB-Pk precipitated from culture supernatants following the addition of ammonium sulphate to a saturation level of 80%, 60%, 50% or 40% (data not shown). From such observations, 50% salt saturation levels were selected and subsequently employed to concentrate LTB-Pk from small scale volumes (<200 ml) of culture medium. Although this strategy was quite successful at concentrating LTB-Pk, the exposure of the culture medium to ammonium sulphate resulted in cleavage of the Pk epitope. This was first suggested when unprecipitated and precipitated samples of LTB-Pk were analysed by SDS-PAGE, where it was shown that precipitated LTB-Pk had a greater electrophoretic motility than unprecipitated LTB-Pk. Confirmation that this difference in the migration of LTB-Pk was due to cleavage of the Pk-epitope was demonstrated by the failure of MAb SV5-P-k to detect precipitated LTB-Pk in Western blot assays. Preliminary attempts to preserve the LTB-Pk fusion by the addition of the metal-ion chelator, EDTA, were based on the observation that *Vibrio sp.*60 secretes a metallo-like protease [Dr. T.R. Hirst, personal communication], and thus the

presence of EDTA could potentially inhibit the protease activity. The fidelity of this strategy was vindicated when samples of precipitated LTB-Pk that had been dialysed against PBS-EDTA buffers, were assessed for the presence of full-length LTB-Pk in both Western blot and immune-precipitation assays. The earlier observations that LTB-Pk could be expressed, intact, in *Vibrio sp.*60 suggested that the Pk sequence did not contain a binding site for any *Vibrio sp.*60 secreted protease. Thus, the susceptibility of LTB-Pk to protease activity following exposure of the culture medium to ammonium sulphate was interesting. It is possible that the protease responsible for the cleavage of the Pk-epitope is a zymogen and becomes activated in the presence of ammonium sulphate. Alternatively, it is possible that ammonium sulphate caused a conformational change or partial unfolding of the LTB-Pk, to reveal the protease binding site, resulting in subsequent cleavage. In either case, the cleavage of the Pk-epitope highlights the problems of using ammonium sulphate to concentrate LTB-Pk, since it involves the inclusion of an additional chemical substance that may influence the efficacy of the final product. In addition, ammonium sulphate precipitation is not a feasible strategy for concentrating LTB-Pk from large volumes (over 1 litre) of culture medium, since it requires extensive quantities of the salt, and is extremely time consuming.

An alternative and more attractive procedure for concentrating LTB-Pk from culture medium was ultrafiltration using a GF15 dialysis membrane with a selective porosity of 10K. The principal advantages over ammonium sulphate precipitation being (1) there was no need to add an extraneous substance that subsequently had to be removed (2) the absence of ammonium sulphate could potentially preserve the LTB-Pk fusion bypassing the need for EDTA and (3) there was little limitation in the volume of culture medium that could be concentrated. Such procedures could be used to extensively concentrate LTB-Pk from culture volumes in excess of 4 litres, the removal of water and small molecules (<10K) was facilitated by recirculating the culture medium through the membrane under pressures of 5-10 lbs/in^2. One interesting observation resulting from the SDS-PAGE analysis of ultrafiltrated LTB-Pk, was that, apart from LTB-Pk, there was only one other protein that could be concentrated to appreciable levels at the same time as LTB-Pk. This suggests that few proteins are constituitively secreted from *Vibrio sp.*60 and thus, supports the notion that

selective secretion of LTB-Pk from *Vibrio sp.*60 could permit easier purification of LTB-Pk, since less protein contaminants have to be removed. Concentration of LTB by ultrafiltration using a cross-flow system and employing polyethersulphone membranes has previously been described [Amin and Hirst, 1994], the yield of LTB recovered from the membranes approaching 73%. In the ultrafiltration procedure described here, it was estimated that 85-90% of LTB-Pk was recovered from the membrane.

As well as concentrating LTB-Pk from culture supernatants, the GF15 dialysis membrane could be utilised to dialyse LTB-Pk *in situ* against the buffer of choice. Such versatility in the use of the GF15 dialysis membranes highlights their attractiveness for incorporation into purification regimes in which the proteins have to be concentrated from large culture volumes prior to purification. In addition to the above advantages, GF15 membranes are extremely economical since they are readily re-usable [personal observations].

4. Purification of LTB-Pk.

LTB has been reported to be purified to homogeneity by direct application of clarified *E.coli* lysates to gel filtration columns containing galactose as a constituent of the gel matrix [Clements and Finkelstein, 1979; Clements *et al.*, 1990]. The purified protein can be subsequently eluted by application of 0.2M galactose to the gel filtration column. This association of LTB with the gel matrix is probably a reflection on the ganglioside binding properties of LTB, since LTB binds to gangliosides (and for that matter certain other sugars) through the lactose derivative, galactose [Sixma *et al.*, 1992]. Initial attempts to purify LTB-Pk using a sephadex G-25 column, the column described in the original assay, were unsuccessful. Although LTB-Pk was seen to bind to the column, the protein failed to elute following application of galactose (data not shown). Interestingly the authors who described the original protocol have also experienced problems in eluting LTB-fusion proteins from such columns [Clements, 1990], the reason for this was unclear. Purification of cholera toxin and LTB from culture supernatants of *Vibrio* species, has also been successfully achieved using GM1-ganglioside affinity columns [Tayot *et al.*, 1980]. Thus, other attempts to purify LTB-Pk were based on the affinity chromatography approach. In the absence of available

GM1-ganglioside an anti-Pk MAb SV5-P-k-linked to sepharose 4B was used instead as the immunoaffinity resin. However, although LTB-Pk clearly interacted with the resin support, protein elution was only achievable after the application of extensively denaturating buffers containing urea, that resulted in both the denaturation of LTB-Pk and the MAb SV5-P-k (data not shown). Such conditions not only necessitated that LTB-Pk had to be re-natured *in vitro*, but could also, potentially lead to the denaturation of the Pk-fusion epitope or other epitopes on LTB-Pk that could be important for the enterotoxin's adjuvanticity. In addition, the degradation of MAb SV5-P-k during the elution step would mean that for each subsequent purifications, new immunoaffinity columns would have to be constructed, which would increase both the time and cost of the purification procedure. Elution of LTB from GM1-ganglioside immunoaffinity columns normally relies on extensive denaturing conditions and in part, this is thought to be a reflection on the pentameric nature of LTB. It is possible that the five Pk-epitopes present on one pentamer of LTB-Pk, resulted in multiple interactions with the anti-Pk antibody, and thus making elution of LTB-Pk more difficult. In addition, LTB-Pk was observed to irreversibly associate with a variety of sepharose-based resins, even in the absence of MAb SV5-P-k, a finding that was not too surprising considering the high affinity LTB-Pk had for the G25-sephadex column, since both columns contain the galactose-derivative, agarose.

From the above findings it was thought that alternative, more conventional purification strategies that did not employ agarose-based resins, may be more successful at purifying LTB-Pk. One such alternative was hydrophobic interaction chromatography, a process that separates biomolecules by their degree of hydrophobicity. The first hydrophobic column investigated was phenyl superose, which had previously been reported to successfully purify LTB to homogeneity from *Vibrio sp.*60 culture medium [Amin and Hirst, 1994]. The elution of LTB from a resin that contains a strongly hydrophilic matrix like agarose, was surprising in light of the previous difficulties with such supports. It is possible that the attachment of the phenyl group to the carbohydrate hindered the interaction of the putative agarose-binding domain of LTB with the agarose-based support. If this was the case, it follows that only hydrophobic interactions would facilitate interaction of LTB-Pk with

phenyl superose, and as such, elution of LTB-Pk from the column could potentially be achieved by conventional means. Ultrafiltrated preparations of LTB-Pk were shown to bind to the phenyl superose under high salt conditions, with the vast majority of the contaminating material, that failed to form sufficient hydrophobic interactions with the phenyl superose, being removed in the early stages of the purification. Application of a decreasing salt concentration resulted in LTB-Pk being eluted at salt levels of approximately 0.65M which is slightly higher than the reported salt concentration for eluting LTB [Amin and Hirst, 1994]. However, unlike the results of Amin and Hirst, LTB-Pk was not eluted as a homogeneous preparation. This was first demonstrated by the evidence of a slight 'bur' in the hydrophobic interaction chromatography profile and confirmed when eluted samples of LTB-Pk were examined by SDS-PAGE. Having said this, the actual level of contamination was small. Apart from the failure to completely purify LTB-Pk, there are additional drawbacks to the use of phenyl superose. For example, the columns are expensive and are normally only employed for the purification of small amounts of protein.

The results shown here suggested that an inexpensive alternative to phenyl superose, is the Macro-prep.® t-butyl hydrophobic support. t-butyl is principally successful at purifying proteins that have intermediate to weakly hydrophobicity, and is chemically stable. Purification of LTB-Pk on this latter hydrophobic matrix, resulted in the same efficiency of purification as that of the phenyl superose-based purification. However, in both instances, hydrophobic interaction chromatography could not purify LTB-Pk completely from *Vibrio sp.*60 protein contaminants.

In contrast to these findings, application of LTB-Pk to the sulphur-based cation exchange matrix and its subsequent elution, resulted in a highly purified preparation of LTB-Pk, with no apparent contaminating material, as determined by SDS-PAGE. Thus in terms of purity, ion-exchange chromatography is superior to hydrophobic chromatography for purifying LTB-Pk from crude culture medium. There are several other advantages to the ion-exchange technique. For example, unlike hydrophobic chromatography where protein samples are applied under high salt conditions, there is no need to introduce and subsequently remove, an extraneous substance (e.g. ammonium sulphate) to the material to be purified.

Further, not only did the absence of ammonium sulphate during the ion-exchange procedure help preserve the Pk fusion, the retention of the oligomerisation and GM1-binding properties of LTB-Pk, suggests that the gentle conditions under which the purification was conducted, did not damage any epitopes that are important for the physiological properties of LTB-Pk. Combining these observations, with the fact that the Bio-rex 70 column is cheap, easy to store, easy to regenerate, readily available, does not need expensive equipment to operate, and is re-usable, highlights its potential to purify LTB-Pk on the industrial level.

5. Systemic Immunogenicity of LTB-Pk.

LTB is a potent systemic (and mucosal) immunogen, and it is thought that this property may contribute to the adjuvanticity of the molecule. Initial studies on the immunogenicity of ammonium sulphate purified LTB-Pk were conducted in the murine Balb/c strain (H-2K^d) which are reported to be high responders to LTB [source of personal communication]. It was suggested from these studies that LTB-Pk alone was a potent systemic immunogen, inducing high levels of serum immunity to itself following intraperitoneal administration. Although, the immunogenicity of LTB-Pk was slightly abrogated by complexing LTB-Pk to the Pk-specific MAb, SV5-P-k, the reduction in the response was slight. There were two interesting observations from these initial studies. The first being that the presence of additional adjuvant like alum, was not apparently necessary to potentiate the immune response to LTB-Pk. Secondly, the immunity generated to LTB-Pk was predominately directed at LTB and not the Pk-epitope. There have been previous reports on the poor serum immunity to the Pk-tag when the epitope was incorporated into immunogenic solid matrix-antibody-antigen (SMAA) complexes [Randall *et al.*, 1993; 1994]. This was thought to be beneficial, since it implied that the immune response was not directed at the irrelevant epitope.

One factor that had to be taken into consideration in these initial studies, was that the LTB-Pk preparation was not completely pure. Thus it was possible that contaminants in the LTB-Pk preparations may be responsible, or contribute, to the potency of LTB-Pk, e.g. by polyclonally activating B cells. However, there was evidence to suggest this was not the

case. Firstly, analysis of the sera from vaccinated mice, demonstrated that the immune response was highly specific for LTB-Pk. Secondly, when later immunisation studies using highly purified LTB-Pk were conducted, very high levels of serum immunity specific for LTB-Pk were detectable (data not shown). This evidence suggests that LTB-Pk, like LTB, is a potent systemic immunogen.

6. Systemic immunogenicity of SIV-MAb-LTB complexes.

Preliminary immunogenicity studies on the efficacy of LTB-Pk to promote immunity to conjugated SIV proteins, were concerned with analysing the immune response to the simian immunodeficiency virus (SIV) recombinant gag gene product, p17 or to denatured SIV proteins from SIV-infect cell lysates. Such studies suggested that the conjugation of recombinant p17 to LTB-Pk via MAb SV5-P-k could potentiate the immune response to recombinant p17 4-8 fold more than immunisation with p17-MAb complexes alone. Further, following immunisation with p17-MAb-LTB complexes the resulting anti-p17 titres, were similar in all immunised mice and also were almost equivalent to the response generated following immunisation with p17-MAb complexes in the presence of alum. The potency of p17-MAb-LTB complexes could be slightly enhanced by the inclusion of a second adjuvant, alum. This difference in response to His-p17-Pk, was not due to variability in doses of administered antigen in the different vaccines, since comparative SDS-PAGE analysis of the various vaccines prior to vaccination, demonstrated that all vaccines contained equivalent amounts of His-p17-Pk and where appropriate LTB-Pk.

In an attempt to characterise the antibody response to His-p17-Pk, the sera was re-assayed for antibodies of the IgG class and divided into Protein A-binding (IgG subclasses of IgG_{2a}, IgG_{2b} and IgG_3) versus non Protein A binding (IgG subclass IgG_1) antibodies. Although all immunogenic vaccines resulted in a demonstrable IgG response (data not shown), only LTB-containing vaccines induced antibodies that could interact with Protein A, suggesting that LTB-Pk can modulate the immune response to His-p17-Pk.

Serum immunity to LTB-Pk was also demonstrable following immunisation with p17-MAb-LTB complexes (either alone or with alum) and this antibody response was readily

detectable with HRP-conjugated Protein A (data not ahown). The presence of IgG antibodies is indicative of the involvement of cell -mediated immunity, since the switch from IgM production to IgG production is governed by the presence of lymphokines [reviewed by Finkelman *et al.*, 1990]. Support for the induction of cell-mediated immune responses, was suggested when the splenocytes from immune mice were investigated *in vitro* for their ability to respond to His-p17-Pk and LTB-Pk. Mixed lymphocyte proliferation assays demonstrated that mice immunised with p17-MAb-LTB complexes in the presence or absence of alum, responded to stimulation with His-p17-Pk with a more enhanced proliferation than mice immunised with p17-MAb complexes adsorbed onto alum. In an attempt to identify proliferating cell type, the phenotype of the proliferating cells were determined by FACscan analysis. Such studies revealed that after a five day stimulation period, the vast proportion of cells detectable by FACscan analysis expressed the CD4 molecule on their cell surface (i.e. the cells were CD4+ T cells), there being little evidence of the presence of B cells. The lack of detectable B cells after a five day stimulation *in vitro* was not due to the absence of B cells in the initial lymphocyte samples since FACscan analysis of the original preparation revealed that an approximately equal number of B cells and $CD4^+$ T cells were present. However, although the evidence would suggest that $CD4^+$ T cells are responding in the assay, it is possible that the B cells died during the culture period, and thus the high level of CD4+T cell detection, was simply due to the $CD4^+$T cells' ability to survive during the culture period. Thus, in a second attempt to determine whether T cells are responding to specific antigenic stimulation, the supernatants from stimulated cultures were assayed for the presence of cytokines.

Based on their production of cytokines murine $CD4^+$ T cell clones have been divided into two groups; T_H1 cells that predominantly produce IL-2, IL-3, lymphotoxin, and IFN-γ or T_H2, producing IL-3, IL-4, IL-5 and IL-6 [Mosmann *et al.*, 1986]. Stimulation of immune cells from p17-MAb/alum, p17-MAb-LTB or p17-MAb-LTB/alum immunised mice with His-p17-Pk, resulted in very little IL-2 being detectable in the culture supernatants. This was not due to insensitivity of the cytokine-ELISA assay to detect IL-2, since high levels of IL-2 were readily detectable in the cultures that had been mitogenically stimulated with PHA.

The lack of detection of IL-2 in antigen-stimulated cultures has been observed by others [A. Mowat, personal communications], the explanation for which, is unknown. In contrast to these findings, high levels of IFN-γ were secreted by His-p17-Pk-specific immune cells from mice vaccinated with p17-MAb-LTB complexes (with or without alum), but IFN-γ production detectable from lymphocytes from mice primed with p17-MAb in association with alum was below background levels. Very minor levels of IL-5 could be detected from each culture, and was highest in the mice vaccinated with p17-MAb-LTB complexes in association with alum. This poor response to IL-5 from spleen cultures was also evident from PHA stimulated cultures, and has been reported by others [Wilson *et al.*, 1991], the reason for which is unknown. This evidence suggests that the presence of LTB-Pk in the vaccine formulations leads to the stimulation of T cells capable of secreting IFN-γ, possibly CD4+ T_H1 cells. Whether, CD4+ T_H2 cells are also stimulated can not be presumed since there is insufficient evidence that a T_H2 profile was present. Similar studies with cholera toxin [Vajdy and Lycke, 1993; Jackson *et al.*, 1993] have shown that intraperitoneal administration of the enterotoxin and unrelated antigen can lead to a characteristic T_H1 response to the unrelated antigen in the spleen, which would support the evidence presented here. However, it is important to point out that, the proliferation assay relied on unfractionated splenic populations. A major disadvantage of such an approach is that the relationship to the cell type producing the cytokine can only be made by indirect argument. In the present study, the FACscan assayed for the presence of the CD4+ T cell subset after antigenic stimulation, and not the $CD8^+$ T cell subset. $CD8^+$ T cell, normally characterised as cytolytic T cells, have now been shown to be capable of secreting a cytokine pattern similar to that of T_H1 cells i.e. they can produce cytokines similar to that of T_H1 cells, like IFN-γ and IL-2 [Fong and Mosmann, 1990]. Thus in can not be ruled out that $CD8^+$ T cells or some other IFN-γ producing cell, were responsible for the high level of IFN-γ production. For future work, the use of purified $CD4^+$ T cells would ensure that the antigen effects noted were due to direct stimulation of $CD4^+$ T cells and not due to stimulation of other cell types that could influence the cytokine pattern observed. In addition, *in situ* hybridisation assays that specifically probe for a certain

cytokine RNA levels in cells that respond to antigen-specific stimulation, will also help to characterise the responding cells further.

These initial immunogenicity studies gave a strong indication on the efficacy of conjugating p17-MAb complexes to LTB-Pk. Unfortunately, they failed to establish whether co-administration of His-p17-Pk with LTB-Pk would similarly potentiate the response to His-p17-Pk. Subsequent experiments that compared the efficacy of co-administration of LTB-Pk with His-p17-Pk as opposed to p17-MAb-LTB complexes to promote immunity to His-p17-Pk, revealed that following three intraperitoneal immunisations, co-administration of His-p17-Pk with LTB-Pk was as efficient as p17-MAb-LTB complexes at potentiating the response to His-p17-Pk (data not shown). This would suggest that the complexing of the recombinant p17 to LTB-Pk via the monoclonal antibody bridge (and therefore the targeting of the complex onto GM1-ganglioside) is not a vital requirement for the LTB-Pk to adjuvant the response to His-p17-Pk. However, a later series of systemic immunogenicity studies using recombinant His-p27-Pk as a target antigen, revealed that although three immunisations with His-p27-Pk co-administered with LTB-Pk enhanced the immune response to His-p27-Pk in a similar manner to that seen following immunisation with p27-MAb-LTB complexes, the p27-MAb-LTB complexes were more potent. This was suggested by the evidence that two administrations of p27-MAb-LTB complexes induced a 8-10 fold enhancement in the response to His-p27-Pk, in comparison to the response generated following co-administration of His-p27-Pk and LTB-Pk. Thus it would seem that, conjugation of His-p27-Pk to LTB-Pk via MAb SV5-P-k leads to a more efficient presentation of recombinant p27 to the immune system. Furthermore, these later studies employed highly purified preparations of LTB-Pk in the vaccine formulations, thus the adjuvant effects mediated by LTB-Pk were not due to impurities in the vaccine preparations.

These results highlight the efficacy of SIV-MAb-LTB complexes as systemic immunogens and suggest that targeting of recombinant SIV antigens onto GM1-ganglioside via LTB-Pk, is important at promoting a more efficient response to the recombinant SIV antigen.

7. Mucosal immunogenicity of SIV-MAb-LTB complexes.

Intranasal administration of 1μg of His-p17-Pk either alone, as part of an immune-complex (p17-MAb), co-administered with LTB-Pk or conjugated to LTB-Pk (p17-MAb-LTB) did not induce any detectable anti-His-p17-Pk specific humoral immunity in the serum or saliva of vaccinated mice. In contrast, both co-administration of His-p17-Pk with LTB-Pk and vaccination with p17-MAb-LTB complexes induced a significant serum antibody response to LTB-Pk demonstrating that LTB-Pk itself to be immunogenic in the nasal mucosae, a finding which was later supported by the identification of anti-LTB-Pk secretory antibody in the saliva of vaccinated mice. LTB, like CTB is thought to increase adsorption of protein antigens over the mucosal epithelium when co-administered with the protein, though this may not always be the case. For LTB-conjugates uptake of LTB by the mucosae epithelium should, in theory result in concomitant uptake of conjugated protein, and thus increase the chance of stimulating mucosal immunity. Thus, it was surprising and disappointing that p17-MAb-LTB complexes did not prime the mucosal system against His-p17-Pk.

Several possibilities exist as to why LTB-Pk failed to potentiate the immune response to His-p17-Pk. Firstly, protease activity in the intranasal cavity may result in the cleavage of the Pk epitope from both LTB-Pk and His-p17-Pk, which would effectively destroy the linkage between the two recombinant antigens, or that His-p17-Pk itself was rapidly degraded. Secondly, the antibody molecule itself, could have been subject to proteolytic attack, once again destroying the linkage of His-p17-Pk and LTB-Pk. Thirdly, the affinity of LTB-Pk for GM1 was reduced due to the linkage of antibody and antigen, and thus complexes did not form a strong interaction with the epithelial cells promoting their uptake.

In an attempt to understand which theory was the most likely explanation, the stabilities of LTB-Pk, MAb SV5-P-k and His-p17-Pk upon exposure to mucosal proteases were investigated *in vitro*. Neither MAb SV5-P-k nor LTB-Pk were shown to be susceptible to proteolytic attack. In contrast, His-p17-Pk was extremely vulnerable to proteolytic attack and could be rapidly degraded upon exposure to mucosal proteases. Although such a result

does not formally prove that breakdown of His-p17-Pk is responsible for the poor immunogenicity exhibited by the His-p17-Pk vaccine formulations, the idea is attractive.

It was therefore decided that only recombinant SIV proteins that were more resilient to proteolytic attack should be used for the investigation into the mucosal immunogenicity of SIV-MAb-LTB complexes. One such recombinant SIV protein was His-p27-Pk, which like His-p17-Pk is a recombinant gag gene product. Proteolysis experiments with His-p27-Pk demonstrated that even at minor concentrations, although some degradation was detectable in Western blot assays, the protein seemed to be fairly resistant to proteolytic degradation. Further, when p27-MAb-LTB complexes were incubated with mucosal proteases, then mixed with *Staphylococcus aureus*, the whole complex was efficiently precipitated, providing additional support that such p27-MAb-LTB complexes are relatively stable and have the potential to be used as mucosal immunogens.

This theory was tested in immunisation studies using Balb/c mice given p27-MAb-LTB intranasally three times. Specific immunity to His-p27-Pk and LTB-Pk was readily detectable by ECL immune assays, in the sera of all vaccinated mice 10 days after the final immunisation. Little of the immune response was directed at the Pk epitope and when the sera was re-assayed for reactivity with Western-blotted viral proteins from SIV-infected cells, antibodies specific for both p27 and its precursor, p57, were identified. Such specific immunity in the sera, suggests that p27-MAb-LTB can be taken up by the nasal mucosae for presentation to mucosal B cells and (presumably) T cells.

His-p27-Pk specific immunity was evident in the lungs of intranasally vaccinated mice (as measured by ELISPOT assays) immunised with p27-MAb-LTB complexes, but no antibodies to His-p27-Pk could detected in the saliva. One of the interesting observations with regards the His-p27-Pk-specific antibody isotype distribution was that the IgG isotype was the dominant isotype in both the lungs and the spleen. High levels of IgA antibodies specific for His-p27-Pk were also readily detectable in the lungs but not in the spleen. Such high levels of IgA specific antibodies in the mucosa and minor levels in the periphery is consistent with mucosal priming of the immune response [Murphy *et al.* 1994]. In viral infections there is normally a correlation between serum IgG and protection against pulmonary virus growth

[Hall *et al.*, 1991; Walsh *et al.*, 1987] and this has been attributed to serum IgG transudating the lung epithelium as opposed to the presence of IgG-secreting cells in the lungs. The data presented here, suggests that IgG-secreting B cells themselves can reside in the lung, though whether the presence of the IgG-secreting cells is a reflection of the trafficking of peripheral IgG-secreting cells into the lung or, whether the IgG-secreting cells are from a mucosal origin, is unknown. Studies by Jones and Ada, 1986 have quantitated the proportion of IgM, IgG and IgA secreting cells in the lungs of mice following virus infection and have noted that the IgG-screting B cells predominate. Whereas, similar studies on the isotype distribution in the small intestine have revealed that, following virus infection, IgA-secreting B cells predominate in the Peyers patches. Such evidence suggests that IgG-secreting cells may have a prominent role in the immune surveillance of the lung against viral infections or that factors in the lung microenvironment favours the generation of IgG-secreting B cells. Thus, the presence of His-p27-Pk-specific IgG-secreting cells in the lungs of p27-MAb-LTB vaccinated mice, may be a reflection on the presence of the viral antigen in the vaccine. Alternatively, the existence of IgG-secreting cells in the lungs, may be the result of an inflammatory response following p27-MAb-LTB administration. This needs further investigation.

Recent evidence by McGhee and colleagues (1993), suggests that in mucosal tissues, T_H2 cells predominate and produce IL-5 which promotes the generation of IgA-secreting cells. Others support this observation [Beagley *et al.*, 1988]. Thus, the presence of IgA-secreting B cells in the lungs of vaccinated mice, may indicate that intranasal administration of p27-MAb-LTB complexes can selectively prime T_H2 cells in the mucosae.

The relevance of secretory IgA in the upper respiratory tract for preventing virus replication has also been recently advocated [Renegar and Small, 1991]. However, there was no evidence that intranasal administration of p27-MAb-LTB complexes induced His-p27-Pk specific IgA in the saliva, and only minor levels of anti-LTB-Pk IgA antibodies were detected. Although this poor response may be a reflection on the time course of the experiment e.g. saliva was collected fairly soon after immunisation (11 days), even at later times (35 days post immunisation) only anti-LTB-Pk antibodies were detected (data not shown). Alternatively, the inability to detect IgA antibodies to His-p27-Pk and the detection

of only low levels of anti-LTB-Pk IgA antibodies may be due to the insensitivity of the ECL-assay performed.

The induction of long term memory, is obviously desirable in the design of vaccines. It has generally been assumed that the mucosal immune system does not display memory. However, this is now disputed [Lycke *et al.*, 1987; Lycke and Holmgren, 1986; 1987; Vajdy and Lycke, 1993]. The ability of intranasal administration of p27-MAb-LTB complexes to induce memory to His-p27-Pk and LTB-Pk was examined in lymphocyte proliferation assays. Immune cells from mice that had been intranasally vaccinated three times with p27-MAb-LTB complexes, were stimulated *in vitro* with His-p27-Pk and LTB-Pk, three months after the last immunisation. Both His-p27-Pk and LTB-P specific immune cells were shown to respond to antigenic challenge. Phenotypic analysis of the lymphocytes following a five day stimulation revealed that, $CD4^+$ T cells predominated in LTB-Pk stimulated cultures. In His-p27-Pk stimulated cultures, although $CD4^+$ T cells tended to predominate, there was also evidence that $CD8^+$ T cells may be responding to antigenic stimulation as well. There was also evidence that B cell memory occurred following intranasal immunisation with p27-MAb-LTB complexes, since serum immunity to the recombinant antigens was detectable following a fourth intranasal boost, three months after the third immunisation (data not shown). Thus, both B-cell and T-cell memory may be generated by intranasal immunisation with p27-MAb-LTB complexes.

One question this study does not determine is whether intranasal co-administration of LTB-Pk and His-p27-Pk would promote similar levels of immunity to the recombinant antigens that were seen following immunisation with p27-MAb-LTB complexes. Preliminary studies have shown that two intranasal immunisations with His-p27-Pk co-administered with LTB-Pk or administration of p27-MAb-LTB complexes, followed by one intraperitoneal immunisation with the respective vaccines, resulted in serum immunity to His-p27-Pk in mice vaccinated with p27-MAb-LTB complexes but not in mice given His-p27-Pk co-administered with LTB-Pk (data not shown). Such findings suggest that the linkage of His-p27-Pk to LTB-Pk is important for the recombinant SIV protein to be immunogenic by the mucosal route i.e. the ability to target His-p27-Pk onto GM1-ganglioside via the binding of LTB-Pk to

the glycolipid, is crucial to promote immunity to the recombinant SIV protein in the mucosal system. In support of this observation, Dertzbaugh *et al.*, 1990, have shown that CTB-peptide fusions that inhibit GM1-ganglioside binding, are not immunogenic by the mucosal route. Nevertheless, it still has to be confirmed that p27-MAb-LTB complexes are more immunogenic by the mucosal route than co-administration of His-p17-Pk and LTB-Pk.

8. Areas of Future Investigation.

One area of future investigation is continuation of the studies into the efficacy of SIV-MAb-LTB complexes as immunogens, particularly their immunogenicity in the mucosal immune system. The mucosal immunogenicity results presented above, have concentrated on p27-MAb-LTB complexes containing ammonium sulphate purified LTB-Pk. It will need to be verified that highly purified preparations of p27-MAb-LTB complexes have similar efficacies in the mucosal system.

Although these studies have centred on the administration of SIV-MAb-LTB complexes by the intranasal route, there have been problems with the administration of the complexes. In some instances the use of diethyl ether as the anaesthetic has lead to fatalities. An approach that is currently under evaluation is the administration of vaccines by nebulisation. In this instance, soluble vaccine formulations are vaporised under high pressure, the vapour is then readily inhaled by the mice. This procedure ensures that the vaccine reaches the lungs and since no anaesthetic is necessary, it is harmless to the experimental animals. Preliminary work with the nebulisation system has concentrated on analysing the ability of highly purified LTB-Pk to induce serum immunity to itself, following intranasal nebulisation. So far, the results have been promising, with mice exhibiting high levels of serum immunity to LTB-Pk following four nebulisations. However, it has still to be established what is the ideal dose of antigen needed for efficient stimulation of the immune system following nebulisation. At present, high doses of antigen are being employed (approximately 50μg of antigen per mouse) since the efficiency rate of antigen uptake by the mice following nebulisation is thought to be as low as 1/10th of the administered dose. Whether nebulisation will be a successful procedure for the administration of SIV-MAb-LTB

complexes remains to be verified. Recent experiments have suggested that administration of the complexes in such a manner, did not lead to serum immunity to the recombinant SIV protein and only low levels of immunity to LTB-Pk. Whether, this poor response to the recombinant antigens was due to damage of the vaccine following vapourisation, or due to too low a dosage, remains to be investigated.

It has recently been established that immunisation with CTB and formalin inactivated influenza A virus can lead to virus specific $CD8^+$ CTL.The level of immunity is similar to natural infection with live replicating virus [Mbawuike and Wyde, 1993]. Considering the important role cytotoxic $CD8^+$ T cells have in the control of virus infections, it would be of interest to establish whether administration of SIV-MAb-LTB complexes can lead to priming of this arm of the immune system.

REFERENCES

ADAMS, M.D., KELLEY, J.M., GOCAYNE, J.D., DUBNICK, M., POLYMERPOULOUS, M.H., XIAO, H. and MERRIL, C.R. (1991). Complementary DNA sequencing expressed tags and the human genome project. *Science* **252**, 1651-1656.

ADAMS, S.E.,DAWSON,K.M.,GULL,K.,KINGSMAN,S.M. and KINGSMAN,A.J. (1987). The expression of hybrid HIV:Ty virus-like particles in yeast. *Nature* **329**, 68-70.

AGGARWAL, A.,KUMAR,S.,JAFFE,R.,HONE,D.,GROSS,M.and SADOFF,J. (1990). Oral *salmonella*: malaria circumsporozoite recombinants induce $CD8^+$ cytotoxic T cells. *J.Exp.Med.* **172**, 1083-1090.

ALDOVINI, A. and YOUNG,R.A. (1991). Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. *Nature* **351**, 479-482.

ALLISON, A.C. and GREGORIADIS,G. (1974). Liposomes as immunological adjuvants. *Nature* **252**, 252.

ALLISON, AC. and BYARS,N.E. (1986). An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and cell-mediated immunity. *J.Immunol.Methods* **95**, 157-168.

ALMEIDA, J.D., RUBENSTEIN, D. and STOTT, E.J. (1971). New antigen-antibody system in Australia-antigen positive Hepatitis. *Lancet*, **ii**, 1225-1227.

ALMOND, J.W.,WESTROP,G.D. and CANN,A.J.(1985). Attenuation and reversion to neurovirulence of the Sabin poliovirus type-3 vaccine. In *Vaccine 85: molecular and chemical basis of resistence to parasitic,bacterial and Viral disease*. Edited by Lerner, R.H., Chanock,R.M., Brown,F., 271-279.

AMIN, T. and HIRST, T.R. (1994). Purification of the B-subunit oligomer of *Escherichia coli* heat-labile enterotoxin by heterologous expression and secretion in a marine *Vibrio*. *Protein Expression and Purification*, **5**, 198-204.

AMIT, A.G.,MARIUZZA,R.A.,PHILLIPS,E.E.V. and POLJAK,R.J. (1986). Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. *Science* **233**, 747-753.

ANDERSSON, M.,PAABO,S.,NILSSON,T. and PETERSON,P.A. (1985). Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell* **43**, 215-222.

ANDREW,M.E.,KARUPIAH,G.,BOYLE,D.B.,BLANDEN,R.V.,MULLBACHER,A.,RAMSHAW,I.A. and COUPER,B.E.H. (1991). Effects of vaccinia virus-expressed IL-2 on the immune system of sublethally irradiated mice. *Micro.Pathol.* **10**, 363-371.

ASENJO, J.A. and PATRICK, I. (1990) *Protein Purification Applications*, eds. Harris, E.L.V. and Angal, S. ORL Press.

BAGDASARIAN, M.M.,AMANN,E.,LURZ,R.,RUCKERT,B. and BAGDASARIAN,M. (1983). Activity of the hybrid *trp-lac* (*tac*) promoter of *Escherichia coli* in the *Pseudomonas putida*. Construction of broad-host-range, controlled-expression vectors. *Gene* **26**, 273-282.

BALKWILL, F.R. and BURKE,F. (1989). The cytokine network. *Immunol.Today* **10**, 299-304.

BARLOW, D.J.,EDWARDS,M.S. and THORNTON,J.M. (1986). Continuous and discontinuous protein antigenic determinants. *Nature* **322**, 747-748.

BARRETT,T.A.,GAJEWSKI,T.F.,DANIELPOUR,D.,CHANG,E.B.,BEAGLEY,K.W., and BLUESTONE,J.A. (1992). Differential function of intestinal intraepithlial lymphocyte subsets. *J.Immunol.* **149**, 1124-1130.

BEAGLEY,K.W.,ELDRIDGE,J.,KIYONO,H.,EVERSON,M.P.,KASPMAN,W.J.,HONS O,T. and MCGHEE,J.R. (1988).Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. *J.Immunol.* **141**, 2035-2042.

BENOIT, J.P.,COURTEILLE,F., and THIES,C.A. (1986). A physiocochemical study of the morphology of progestrone-loaded poly(D-L-lactide) microspheres. *Int.J.Pharm.* **29**, 95-102.

BENVENISTE, R.E., ARTHUR, L.O., TSAI, C.C, SOWDER, R., COPELAND, T.D., HENDERSON, L.E., and OROSZLAN, S. (1986). Isolation of a lentivirus from a macaque with lymphoma. Comparison with HTLV-III/LAV and other lentiviruses. *J.Virol.* **60**, 483-490.

BERNSTEIN, A., FILGUEIRA,M.P., SCHUDEL,A., ZAMORANO,P., BORCA,M.and SADIR,A. (1993). Avridine and LPS from Brucella ovis: effect on the memory induced by foot-and-mouth disease virus vaccination in mice. *Vaccine* **11**, 1295-1301.

BERNARD, O.,HOZUMI,N. and TONEGAWA,S. (1978). Sequences of mouse immunoglobulin light chain genes beofre and after somatic changes. *Cell* **15**, 1133-1144.

BERNSTEIN, J.M.,YAMANAKA,N.and NADAL,D. (1994). Immunobiology of the tonsils and adenoids. In *Handbook of Mucosal Immunology*. Edited by PL Ogra, San Diego: Academic Press Inc. 625, 640.

BEVERLEY, P.C.L. (1990). Human T cell memory. *Curr.Top.Microbiol.Immunol.* **159**, 111-122.

BIKOFF, E.K.and ECKHARDT,L.A. (1989). Presentation of IgG2a antigens to class II-restricted T cells by stably transfected B lymphoma cells. *Eur.J.Immunol.* **19**, 1903-1909.

BIRNBOIM, H.C. and DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, **7**, 1513-1523.

BISHOP, R.,LUND,J.,CIPRIANI,E.,UNICOMB,L.and BARNES,G. (1990). Clinical serological and intestinal immune responses to rotavirus infection of humans. In Medical Virology 9 (L.M.de la Maza and E.M.Peterson eds.) 85-110.

BJORKMAN,P.J.,SAPER,M.A.,SAMRAOUI,B.,BENNETT,W.S.,STROMINGER,J.L. and WILEY,D.C. (1987a). Structure of the human class I histocompatibity antigen, HLA-A2. *Nature* **329**, 506-512.

BJORKMAN,P.J.,SAPER,M.A.,SAMRAOUI,B.,BENNETT,W.S.,STROMINGER,J.L. and WILEY,D.C. (1987b). The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**, 512-518.

BLANDEN,R.V.,DOHERTY,P.C.,DUNLOP,M.B.C.,GARDNER,I.D.,ZINKERNAGEL, R.M. and DAVID,C.S. (1975). Genes required for cytotoxicity against virus-infected target cells in K & D redions of the H-2 complex. *Nature* **254**, 269-270.

BLUNDELL, T.L.,SIBANDA,B.L.,STERNBERG,M.J.E. and THORNTON,J.M. (1987). Knowledge-based predicition of protein structures and the design of novel molecules. *Nature* **326**, 347-352.

BOCKMAN, D. and COOPER,M.D. (1973). Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix and Peyer's patches: and electron microscopic study. *Am.J.Anat.* **136**, 455-477.

BODIAN, D. and NATHANSON,N. (1960). Inhibitory effects of passive antibody on virulent poliovirus excretion and on immune response in chimpanzees. *Bull.Johns Hopskins Hosp.* **107**, 143-162.

BOHLER-SOMMEREGGER, K. and LINDEMAYR,H. (1986). Contact sensitivity to aluminium. *Contact Dermatitis* **15**, 295-297.

BOLIVAR,F., RODRIGUEZ,R.L., GREENE,P.J.,BETLACH,M.C.,HEYNECKER,H.L., BOYER,H.W., CROSA,J.H. and FALKOW,S. (1977). Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95.

BOMFORD, R. (1980). The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, *corynebacterium parvum*, *Bordetella pertussis*, muramyl dipeptide and saponin. *Clin.Exp.Immunol.*, **39**, 435-441.

BONNEVILLE,M.,JANEWAY,C.A.JR.,ITO,K.,HASER,W.,ISHIDA,I.,NAKANISHI,N and TONEGAWA,S. (1988). Intestinal intraeptihelial lymphocytes are a distinct set of γδ T cells. *Nature* **336**, 479-481.

BOOG, C.J.P.,NEEFJES,J.J.,BOES,J.,PLOEGH,H.L. and MELIEF,C.J.M. (1989). Specific immune responses restored by alteration in carbohydrate chains of surface molecules on antigen-presenting cells. *Eur.J.Immunol.* **19**, 357-352.

BOURGAULT, I.,GOMEZ,A.,GOMRAD,E.,PICARD,F. and LEVY,J.P. (1989). A virus specific $CD4^+$ cell-mediated cytolytic activity revealed by $CD8^+$ cell elimination regularly develops in uncloned human antiviral cell lines. *J.Immunol.* **142**, 252-256.

BRACK, C.,HIRAMA,M.,LENHARD-SCHULLER,R. and TONEGAWA,S. (1978). A complete immunoglobulin gene is created by somatic rearrangement. *Cell* **15**, 1-14.

BRANDTZAEG, P.,BOSNES,V.,HALSTENSEN,T.S.,SCOTT,H.,SOLLID,L.M. and VALNES,K.N. (1989). T lymphocytes in human gut epithelium preferentially express the α/β antigen receptor and are often CD45/UCH1-positive. *Scand.J.Immunol.* **30**, 123-128.

BRANDTZAEG,P.,SOLLID,L.,THRANE,P.,KVALE,D.,BJERKE,K.,SCOTT,H.,KETT, K.and ROGNUM,T.O. (1988). Lymphoepithelial interactions in the mucosal immune system. *Gut* **29**, 1116-1130.

BROMANDER, A.,HOLMGREN,J.and LYCKE,N. (1991). Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. *J.Immunol.* **146**, 2908-2914.

BROWN,J,H.,JARDETZKY,T.,SAPER,M.A.,SAMRAOUI,B.,BJORKMAN,P.J., and WILEY,D.C. (1988). A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature* **332**, 845-850.

BROWN,J.H., JARDETZKY, T.S., GORGA, J.C., STERN, L.J., URBAN, R.G., STROMINGER, J.L. and WILEY, D.C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature*, **346**, 33-39.

BROWNE, H.,SMITH,G.,BECK,S., and MINSON,T. (1990). A complex between the MHC class I homologue encoded by human cytomegalovirus and β2-microglobulin. *Nature* **347**, 770-772.

BULLER, R.M.L.,SMITH,G.L.,CREMER,K.,NOTKINS,A.L. and MOSS,B. (1985). Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine-negative phenotype. *Nature* **317**, 813-815.

BURKE, K.L.,DUNN,G.,FERGUSON,M.,MINOR,P.D., and ALMOND,J.W. (1988). Antigen chimaeras of poliovirus as potential new vaccines. *Nature* **332**, 81-82.

BURLINGTON, D.B.,CLEMENTS,M.L.,MEIKLEJOHN,G.,PHELAN,M. and MURPHY,B.R. (1983). Haemagglutinin specific antibody responses in the IgG, IgA and IgM isotypes as measured by ELISA after primary or secondary infection of man with influenza A virus. *Infect.Immun.* **41**, 540-545.

BURNET, F.M. (1968). Measles as an index of immunological function. *Lancet* **ii**, 610-613.

BUTLER, N.R.,WILSON,B.D.R.,BENSON,P.F.,DUDGEON,J.A.,UNGAR,J. and BEALE,A.J. (1962). Effect of aluminium phosphate on antibody response to killed poliomyelitis vaccine. *Lancet* **ii**, 114-116.

BYRNE, J.A. and OLDSTONE,M.B.A. (1984). Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomenigitis virus: clearance of virus in vivo. *J.Virol.* **51**, 682-686.

CANNON, M.J.,OPENSHAW,P.J.M. and ASKONAS,B.A. (1988). Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J.Exp.Med.* **168**, 1163-1168.

CARAYANNIOTIS, G. and BARBER,B.H. (1987). Adjuvant-free IgG responses induced with antigen coupled to antibodies against class II MHC. *Nature* **327**, 59-61.

CHAKRABARTI, L., GUYADER, M., ALIZON, M., DANIEL, M.D., DeROSIERS, R.C., TIOLLAIS, P. and SONIGO, P. (1987). Sequence of simian immunodeficiency virus from macaques and its relationship to other human and simian retroviruses. *Nature*, **328**, 543-547.

CHALLACOMBE, S.J. and TOMASKI,T.B. (1987). Model systems of antigen handling. In *Food Allergy and Intolerance*, Edited by J.Brostoff and S.J. Challacombe, 269-285.

CHANTLER, J.K.,FORD,D.K. and TINGLE,A.J. (1981). Rubella-associated arthritis: rescue of rubella virus from peripheral blood lymphocytes two years postvaccination. *Infect.Immunol.* **32**, 1274-1280.

CHEDID, L.,AUDIBERT,F.,LEFRANCIER,P.,CHOAY,J. and LEDERER,E. (1976). Modulation of the immune response by a synthetic adjuvant and analogues. *Proc.Natl.Acad.Sci.USA* **73**, 2472-2475.

CHEN, K.-S.,BURLINGTON,D.B. and QUINNAN,G.V. (1987). Active synthesis of haemaglutinin-specific immunoglobulin A by lung cells of mice that were immunized intragastrically with inactivated influenza virus vaccine. *J.Virol.* **61**, 2150-2154.

CHOTHIA, C.,BOSWELL,D.R.and LESK,A.M. (1988). The outline structure of the T cell alpha and beta receptor. *EMBO J.* **7**, 3745-3755.

CLARK, C.J.,WILSON,A.D.,WILLIAMS,N.W.AND STOKES,C.R. (1991). Mucosal priming of T-lymphocytes responses to fed protein antigens using cholera toxin as an adjuvant. *Immunology* **72**, 323-328.

CLARK, E.A. and LEDBETTER, J.A. (1994). How B and T cells talk to each other. *Nature*, **367**, 425-428.

CLARKE, B.E.,BROWN,A.L., GRACE, K.G., HASTINGS, G.Z., BROWN, F., ROWLANDS, D.J., and FRANCIS, M.J. (1990). Presentation and immunogenicity of viral epitopes on the surface of hybrid hepatitis B virus core particles produced in bacteria. *J.Gen.Virol.* **71**, 1109-1117.

CLARKE,B.E.,NEWTON,S.E.,CARROLL,A.R.,FRANCIS,M.J.,APPLEYARD,G.,SYR ED,A.D., and HIGHFIELD,P.E (1987). Impaired immunogenicity of a peptide epitope after fusion to Hepatitis B core protein. *Nature* **330**, 381-384.

CLAVERIE, J.M.,PROCHNICKA,C.A. and BOUGUELERT,L. (1989). Implications of a Fab-like structure for the T cell receptor. *Immunol.Today* **10**, 10-14.

CLEMENTS, J.D. (1990). Construction of a non-toxic fusion peptide for immunisation against *Escherichia coli* strains that produce heat labile and heat stabile enterotoxins. *Infect.Immun.* **58**, 1154-1166.

CLEMENTS, J.D. and CARDENAS, L. (1993). Development of mucosal protection against the heat stabile enterotoxin (ST) of *Escherichia coli* by oral immunisation with a genetic fusion delivered by a bacterial vector. *Infect.Immun.* **61**, 4629-4636.

CLEMENTS, J.D. and FINKELSTEIN,R.A. (1979). Isolation and Characterization of Homogeneous Heat-Lablie Enterotoxins with High Specific Activity from Escherichia coli Cultures. *Infect.Immun.* **24**, 760-769.

CLEMENTS, J.D.,HARTZOG,N.M. and LYON,F.L. (1988). Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* **6**, 269-277.

CLEMENTS, M.L.,BETTS,R.F.,TIERNEY,E.L. and MURPHY,B.R. (1986). Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. *J.Clin.Microbiol.* **24**, 157-160.

COFFIN, J.M. (1986). Genetic variation in AIDS viruses. *Cell*, **46**, 1-42.

COGNE, M.,BALLET,J.J.,SCHMITT,C. and BIZZINI,B. (1986). Total and IgE antibody levels following booster immunisation with aluminium adsorbed and non-adsorbed tetanus toxoid in humans. *Ann.Allergy* **54**, 148-151.

COHEN, S.,YOSHIOKA,T.,LUCARELLI,M.,HWANG,L.H. and LANGER,R. (1991). Controlled delivery systems for proteins based on poly(lactic/glycolic) microspheres. *Pharm.Res.* **8**, 713-720.

COLIGAN, J.E.,KINDT,T.J.,UEHARA,H.,MARTINKO,J. and NATHENSON,S.G. (1981). Primary structure of a murine transplantation antigen. *Nature* **291**, 35-44.

COULSON,B.S.,GRIMWOOD,K.,MASENDYCZ,P.J.,LUND,J.S.,MERMELSTEIN,N. and BISHOP,R.F. (1990). Comparisons of rotavirus IgA conversion with other indexes of rotavirus infection in a longtitudnal study in childhood. *J.Clin.Microbiol.* **28**, 1367-1374.

COUTELIER, J.P.,VAN DER LOGT,J.T.M.,HEESEN,F.W.A.,WARNER,G., and VAN SNICK J. (1987). IgG_{2a} restriction of murine antibodies elicited by viral infections. *J.Exp.Med.* **165**, 64-69.

CRAIG, S.W. and CEBRA,J.J. (1971). Peyer's patches: an enriched source of precursors for IgA producing immunocytes in the rabbit. *J.Exp.Med.* **134**, 188.

CRESSWELL, P. (1987). Regulation of HLA class I and class II antigen expression. *British Medical Bulletin* **43**, 66-80.

CZERKINSKY, C., NILSON, L.A., NYGREN, H., OUCHTERLONY, O. and TARKOWSKI, A (1983). A solid-phase enzyme-linked immunospot (ELISPOT) assay for the enumeration of specific antibody secreting cells. *J.Immunol.Methods* **65**, 109-121.

DALLAS, W.S. and FALKOW, S. (1980). Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature* **288**, 499-501.

DANIEL, C.,LACROIX,M. and TALBOT,P.J. (1994). Mapping of linear antigenic sites on the S glycoprotein of a neurotropic murine corocavirus with synthetic peptides: a combination of nine prediction algorithms fails to identify relevant epitopes and peptide immunogenicity is drastically influenced by the nature of the protein carrier. *Virology* **202**, 540-549.

DANIEL, M.D., LETVIN, N.L., KING, N.W., KANNAGI, M., SEHGAL, P.K., HUNT, R.D., KANKI, P.J., ESSEX, M. and DeROSIERS, R.C. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science*, **228**, 1202-1204.

DAVENPORT, F.M.,HENNESSY,A.V.and ASKIN,F.B. (1968). Lack of adjuvant effect of Al_2PO_4 on purified influenza virus haemagglutinins in man. *J.Immunol.* **100**, 1139.

DAVIS, M.M. (1990). T cell receptor gene diversity and selection. *Ann.Rev.Biochem.*. **59**, 475-496.

DeVRIES, P.,VAN BINNENDIJK,R.S.,VAN DER MAREL,P.,VAN WEZEL,A.L.,VOORMA,H.O.*et al.* (1988). Measles virus fusion protein presented in an immunostimulatory complex (ISCOM) induces haemolysis-inhibiting and fusion-inhibiting antibodies, virus specific T-cells and protection in mice. *J.Gen.Virol.* **69**, 549-559.

DEDIEU, J.F.,RONCA,J.,VANDERWERF,S.,HOGLE,J.M.,HENIN,Y., and GIRARD,M. (1992). Polivirus chimaeras expressing sequences from the principal neutralising domain of HIV type 1 virus. *J.Virol.* **66**, 3161-3167.

DEL PRETE, G.F.,CARLI,M.D.,RICCI,M. and ROMAGNANI,S. (1991a). Helper activity for immunoglobulin synthesis of T helper type 1 (TH1) and T helper type 2 (TH2) human T cell clones: The help of TH1 clones is limited by their cytolytic capacity. *J.Exp.Med.* **174**, 809-813.

DEL PRETE,G.F.,DECARLI,M.,MASTROMAWO,C.,BIAGIOTTI,R.,MACCHIA,D., FALAGIANI,P.,RICCI,M. and ROMAGNANI,S. (1991b). Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of *Toxicara carnis* expand *in vitro* human T cells with stable and opposite (type 1 T helper and type 2 T helper) profile of cytokine production. *J.Clin.Invest.* **88**, 346-350.

DEMOTZ, S.,GREY,H.M., and SETTE,A. (1990). The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249**, 1028-1030.

DESROSIERS, R.C., KODEMA, T., RINGLER, D.J., ARTHUR, L.O., SEHGAL, P.K., LETVIN, N.L., KING, N.W. and DANIEL, M.D. (1989). Vaccine protection against simian immunodeficiency virus infection. *Proc.Natl.Acad.Sci.USA* **86**, 6353-6357.

DERTZBAUGH, M.T., PETERSON, D.L., and MACRINA, F.L. (1990) Cholera toxin B-subunit gene fusion: Structural and functional analysis of the chimeric protein. *Infect.Immun.*, **58**, 70-79.

DIMARZO-VERONESE, F., COPELAND, T.D., DeVICO, A.L., RAHMAN, R., OROSZLAN, S., GALLO, R.C. and SARNGARDHARAN, MG. (1986). Characterisation of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III-LAV. *Science*, **231**, 1289-1291.

DOHERTY, P.C.,ALLAN,W. and EICHELBERGER,M. (1992). Roles of $\alpha\beta$ and $\gamma\delta$ T cell subsets in viral immunity. *Ann.Rev.Immunol.* **10**, 123-151.

DOHERTY, P.C.,BIDDISON,W.E.,BENNICK,J.R. and KNOWLES,B.B. (1978). Cytotoxic T-cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. *J.Exp.Med.* **148**, 534.

DOHERTY,P.C., HOU, S. and TRIPP, R.A. (1994). CD8+ T cell memory to viruses. *Curr.Opin.Immunol.*, **6**, 545-552.

DOMB, A. and LANGER,R. (1987). Polyanhydrides. I. Preparation of high molecular weight polyanhydrides. *J.Poly.Sci.* **25**, 3373-3386.

DOMINGUEZ, P., VELASCO, G., BARROS, F. and LAZO, P.S. (1987). Intestinal brush border membranes contain regulatory subunits of adenyl cyclase. *Proc.Natl.Acad.Sci.USA.* **84**, 6965-6969.

DOYLE, C. and STROMINGER,J.L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330**, 256-259.

EAGER,K.B.,HACKETT,C.J.,GERHARD,W.U.,BENNINK,J.,EISENLOHR,L.C.,YEW DELL,J. and RICCIARDI,R (1989). Murine cell lines stably expressing the influenza virus haemaglutinin gene introduced by a recombinant retrovirus vector are constitutive targets for MHC class I- and class II-restricted T lymphocytes. *J.Immunol.* **143**, 2328-2335.

EARLY, P.,HUANG,H.,DAVS,M.,CALAME,K. and HOOD,L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H. *Cell* **19**, 981-992.

ELSON, C.O. and EALDING,W. (1984). Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. *J.Immunol.* **133**, 2892-2897.

ELSON, C.O. and EALDING,W. (1987). Ir gene control of the murine secretory IgA response to cholera toxin. *Eur.J.Immunol.* **17**, 425-428.

ERNST, P.B.,BEFUS,A.D. and BIENSTOCK,J. (1985). Leukocytes in the intestinal epithelium: an unusual immunological compartment. *Immunol.Today* **6**, 50-55.

EVANS,D.J.,MCKEATING,J.,MEREDITH,J.M.,BURKE,K.L.,KATRAK,K.,JOHN,A., FERGUSON,M., and MINOR,P (1989). An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralising antibodies. *Nature* **339**, 385-388.

EWAN, P.W. and LACHMAN,P.J. (1982). In *Clinical Aspects of Immunology*, volume 2, pp.1142-1514, Blackwell Scientific Publications.

FAGBAMI, A.,HALSTEAD,S.B.,MARCHETTE,M. and LARSEN,K. (1988). Heterologous flavivirus infection-enhancing antibodies in sera of Nigerians. *Am.J.Trop.Med.* **38**, 205-207.

FALK, K.,ROTZSCHKE,O.,STEVANOVIC,S.,JUNG,G. and RAMMENSEE,H-G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290-296.

FEARON, D.T. (1993). The CD19-CR2-TAPA-1 complex, CD45 and signalling by the antigen receptor of B lymphocytes. *Current Opinion In Immunology* **5**, 341-348.

FINKELMAN, F.D.,KATONA,I.M.,MOSMANN,T.R. and COFFMAN,R.L. (1988). Interferon-gamma regulates the isotypes of immunoglobulin secreted during *in vivo* humoral immune responses. *J.Immunol.* **140**, 1022-1027.

FINKELMAN, F.D., HOLMES, J., KATONA, I.M., URBAN, J.F.Jr., BECKMANN, M.P., PARK, L.S., SCHOOLEY, K.A., COFFMAN, R.L., MOSMANN, T.R. and PAUL, W.E. (1990). Lymphokine control of *in vivo* immunoglobulin isotype selection. *Ann.Rev.Immunol.* **8**, 303-333.

FONG, T.A.T. and MOSMANN,T.R. (1990). Alloreactive CD8+ T cell clones secrete the TH1 pattern of cytokines. *J.Immunol.* **144**, 1744-1752.

FRANCHINI, G., GURGO, C., GUO, H.C, GALLO, R.C., COLLALTI, E., FARGNOLI, K.A., HALL, L.F., WONG-STAAL, F. and REITZ, M.S.Jr. (1987). Sequence of SIV and its relationship to HIV. *Nature*, **328**, 539-543.

FRANCIS, M.J.,FRY,C.M.,ROWLANDS,D.J. and BROWN,F. (1988). Qualitative and quantitative differences in the immune responses to foot-and-mouth disease virus antigens and synthetic peptides. *J.Gen.Virol.* **69**, 2483-2491.

FRANCIS,M.J.,HASTINGS,G.Z.,BROWN,A.L.,GRACE,K.G.,ROWLANDS,D.J.,BRO WN,F., and CLARKE,B.E. (1990). Immunological properties of hepatitis B core antigen fusion proteins. *Proc.Natl.Acad.Sci.USA* **87**, 2545-2549.

FRIEDE,M.,MULLER,S.,BRIANDE,J.P.,PLAUE,S.,FERNANDES,I.,FRISCH,B.,SCH UBER,F.and VAN REGENMORTEL, M.H.V. (1994). Selective induction of protection against influenza virus infection in mice by a lipid-peptide conjugate delivered in liposomes. *Vaccine* **12**, 791-797.

FULGINITI, V.A.,ELLER,J.J.,DOWNIE,A.W. and KEMPE,C.H. (1967). Atypical measles in children previously immunised with inactivated measles virus vaccine. Altered reactivity to measles virus. *JAMA* **202**, 1075-1080.

FYNAN, E.F., WEBSTER, R.G., FULLER, D.H., HAYNES, J.R., SANTORO, J.C. and ROBINSON, H.L. (1993). DNA vaccines: Protective immunisations by parental, mucosal and gene-gun inoculations. *Proc.Natl.Acad.Sci.USA*. **90**, 11478-11482

GABERT, J.,LANGLET,C.,ZAMOYSKA,R.,PARNES,J.R. and SCMITT-VERHULST,A.-M. (1987). Reconstitution of the MHC class I specificity by transfer of the T cell receptor and Lyt-2 genes. *Cell* **50**, 545-555.

GARRETT, T.P.J.,SAPER,M.A.,BJORKMAN,P.J.,STROMINGER,J.L. and WILEY,D.C. (1989). Specificity pockets for the side chains of peptide antigens in HLA-Aw68. *Nature* **342**, 692-696.

GASSELIN,E.J.,WARDELL,R.,GASSELIN,D.R.,ALTER,N.,FISHER,J.L., and GUYRE,P.M. (1992). Enhanced antigen presentation using human Fcγ receptor (monocyte/macrophage) specific immunogens. *J.Immunol.* **149**, 3477-3481.

GAY,D.,MADDON,P.,SEKALY,R.,TALLE,M.A.,GODFREY,M.,LONG,E., GOLDSTEIN,G.,CHESS,L., and AXEL, A. (1987). Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* **328**, 626-629.

GERMAIN, R.N. (1986). The ins and outs of antigen processing and presentation. *Nature* **322**, 687-689.

GILL,D.M., CLEMENTS, J.D., ROBERTSON, D.C. and FINKELSTEIN, R.A. (1981). Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin . *Infect.Immunol.*. **33**, 677-682.

GIVOL, D.,ZAKUT,R.,EFFRON,K.,REHAVI,G.,RAM,D., and COHEN,J.B. (1981). Diversity of the germ-line immunoglobulins-VH genes. *Nature* **292**, 426-430.

GLENNY, A.T.,POPE,C.G.,WADDINGTON,H. and WALLACE U. (1926). The antigenic value of toxoid precipitated by potassium alum. *J.Pathol.Bacteriol.* **29**, 38.

GOBET, R.,CERNEY,A.,RUEDI,E.,HENGARTNER,H. and ZINKERNAGEL,R.M. (1988). The role of antibodies in natural and acquired resistance to vesicular stomatitis virus. *Exp.Cell.Biol.* **56**, 175-180.

GOOD,M.F.,POMBO,D.,LUNDE,M.N.,MALOY,W.L.,HALENBECK,R.,KOTHS,K.,MI LLER,L.H. and BERZOFSKY,J.A. (1988). Recombinant human IL-2 overcomes genetic nonresponsiveness to malari sporozoite peptides. *J.Immunol.* **141**, 972-977.

GOTO, N.,KATO,H.,MAEYAMA,J-I.,ETO,K. and YOSHIHARA,S. (1993). Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. *Vaccine* **11**, 914-918.

GOUGH, N.M. and BERNARD,O. (1981). Sequences of the joining region genes for Ig heavy chains and their role in the generation of antibody diversity. *Proc.Natl.Acad.Sci.USA* **78**, 509-513.

GRAY, D.,KOSCO,M. and STOCKINGER,B. (1991). Novel pathways of antigen presentation for the maintenance of memory. *International Immunology* **3**, 141-148.

GRAY, D. and MATZINGER,P. (1991). T cell memory is short-lived in the absence of antigen. *J.Exp.Med.* **174**, 969-974.

GRAY,D. and SKARVALL, H. (1988). B cell memory is short-lived in the absence of antigen. *Nature*, **336**, 70-73.

GUPTA, R.K.,RELYVELD,E.H.,LINBLAD,E.B.,BIZZINI,B.,BEN-EFRAIM,S. and GUPTA,C.K. (1993). Adjuvants-a balance between toxicity and adjuvanticity. *Vaccine* **11**, 293-306.

GUY-GRAND, D. and VASSALLI,P. (1993). Gut intraepithelial T lymphocytes. *Current Opinion In Immunology* **5**, 247-252.

GUY-GRAND, D.,MALASSIS-SERIS,M.,BRIOTTET,C. and VASSALLI,P. (1991). Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocytes is induced locally, Correlation between functional assays, presence of perforin and granzyme transcripts and cytoplasmic granules. *J.Exp.Med.* **173**, 1549-1552.

HALL, C.B., WALSH, E.E., LONG, C.E. and SCHNABEL, K.C. (1991). Immunity to and frequency of reinfection with respiratory syncytial virus. *J.Infect.Dis.*, **99**, 693-698.

HALSTEAD, S.B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science* **239**, 476-481.

HANKE, T.,BOTTING,C.,GREEN,E.A.,SZAWLOWSKI,P.W.,RUD,E. and RANDALL,R.E. (1994). Expression and purification of nonglycosylated SIV proteins, and their use in induction and detection of SIV-specific immune responses. *AIDS Res.Hum.Retro.* **10**, 653-662.

HANKE, T. and RANDALL, R.E.R. (1994). Processing of viral proteins for presentation by molecules of the major histocompatibility complex. *Rev.Med.Viriol.* **4**, 47-61.

HANKE, T.,SZAWLOWSKI,P. and RANDALL,R.E. (1992). Construction of solid matrix-antibody-antigen complexes containing simian immunodeficiency virus p27 using tag-specific monoclonal antibody and tag-linked antigen. *J.Gen.Virol.* **73**, 653-660.

HARDING, C.V. and UNANUE,E.R. (1990). Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* **346**, 574-576.

HARDY, S.J.S.,HOLMGREN,J.,JOHANSSON,S.,SANCHEZ,J. and HIRST,T.R. (1988). Coordinated assembly of multisubunit proteins:oligomerisation of bacterial enterotoxins in vivo and in vitro. *Proc.Natl.Acad.Sci.USA* **85**, 7109-7113.

HAREL-BELLAN,A.,QUILLET,A.,MARCHIOL,C.,DEMARS,R.,TURSZ,T., and FRADELIZI,D. (1986). Natural killer susceptibility of human cells may be regulated by genes in the HLA region on chromosome 6. *Proc.Natl.Acad.Sci.USA* **83**, 5688-5692.

HEATH, T.D.,EDWARDS,D.C.,COLLEY,C.M. and RYMAN,B.E. (1976). The adjuvant properties of liposomes. *Biochem.Soc.Trans.* **4**, 129.

HILL, A.B.,MULLBACHER,A. and BLANDEN,R.V. (1993). Ir genes, peripheral cross-tolerance and immunodominance in MHC class I-restricted T-cell responses: an old Quagmire revisted. *Imm.Rev.* **133**, 75-91.

HILLEMAN, M.R. (1985). Newer directions in vaccine development and utilization. *J.Infect.Dis.* **151**, 407-419.

HILSCHMANN, N. and CRAIG,L.C. (1965). Amino acid sequence studies with Bence-Jones proteins. *Proc.Natl.Acad.Sci.USA* **53**, 1403-1409.

HIRABAYASHI,Y.,KURATA,H.,FUNATO,H.,NAGAMINE,T.,AIZAWA,C.,TAMURA, S.-I.,SHIMADA,K. and KURATA,T. (1990). Comparison of intranasal inoculation of influenza HA vaccine combined with cholera toxin B subunit with oral or parental vaccination. *Vaccine* **8**, 243-249.

HIRABAYASHI, Y.,TAMURA,S.I.,SHIMADA,K. and KURATA,T. (1992). Involvement of antigen-presenting cells in the enhancement of the *in vitro* antibody responses by cholera toxin B subunit. *Immunology* **75**, 493-498.

HIRST, T.R.,SANCHEZ,J.,KAPER,J.B.,HARDY,S.J.S. and HOLMGREN,J. (1984). Mechanism of toxin secretion by Vibrio cholerae investigated in strains harbouring plasmids that encode heat-labile enterotoxins of Escherichia coli. *Proc.Natl.Acad.Sci.USA* **81**, 7752-7756.

HOISETH, S.K.and STOCKER,B.A.D. (1981). Aromatic-dependent Salmonella typhimurium are non virulent and effective as live oral vaccines. *Nature* **291**, 238-239.

HOLMGREN, J. (1973). Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural toxoid. *Infect.Immunol.* **8**, 851-859.

HOLMGREN, J., LINDBLAD, M., FREDMAN, P., SVENNERHOLM, L., and MYRVOLD, H. (1985). Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. *Gastroenterology*, **89**, 27-35

HOOFNAGLE, J.H., GERETY, R.J. and BARKER, L.F. (1973). Antibody to Hepatitis-B virus core in man. *Lancet*, **ii**, 869-873.

HORVAT, B.,LOUKIDES,J.A.,ANADAN,L.,BREWER,E. and FLOOD,P.M. (1991). Production of interleukin 2 and interleukin 4 by immune $CD4^{-}CD8^{+}$ and their role in the generation of antigen-specific cytotoxic T cells. *Eur.J.Immunol.* **21**, 1863-1871.

HOZUMI, N. and TONEGAWA,S. (1976). Evidence for somatic rearrangment of immunoglobulin genes coding for variable and constant regions. *Proc.Natl.Acad.Sci.USA.* **73**, 3628-3632.

HU, S.L.,KOSOWSKI,S.G. and DALRYMPLE,J.M. (1986). Expression of AIDS virus envelope gene in recombinant vaccinia virus. *Nature* **320**, 537-540.

HUDSON, L. and HAY, F.C. (1980). In *Practical Immunology*. Blackwell Scientific Publications.

ICHIGE, A.,OISHI,K.and MIZUSHIMA,S. (1988). Isolation and characterisation of mutants of a marine *Vibrio* strain that are defective in production of extracellular proteins. *J.Bacteriol.* **170**, 3537-3542.

JACKSON, R.J.,FUJIHASHI,K.,XU-AMANO,J.,KIYONO,H.,ELSON,C.O. and McGHEE, J.R. (1993). Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxoid by use of cholera toxin as an adjuvant. *Infect.Immun.* **61**, 4272-4279.

JACOBS, W.R.,TUCKMAN,M. and BLOOM,B.R. (1987). Introduction of foreign DNA into mycobacteria using a shuttle plasmid. *Nature* **327**, 532-535.

JACOBSON,S.,RICHERT,J.R.,BIDDISON,W.E.,SATINSKY,A.,HARTZMAN,R.J., and MCFARLAND,H.F. (1984). Measles virus-specific T4+ human cytotoxic T cell clones are restricted by class II HLA antigens. *J.Immunol.* **133**, 754-757.

JACOBSON, S.,SEKALY,R.P.,JACOBSON,C.L.,MCFARLAND,H.F. and LONG,E.O. (1989). HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. *J.Virol.* **63**, 1756-1762.

JANEWAY, C.A.. JONES, B. and HAYDAY, A.C. (1988). Specificity and function of T cells bearing $\gamma\delta$T cell receptors. *Immunol. Today.* **9**, 73-76

JARAQUEMADA, D.,MARTI,M. and LONG,E.O. (1990). An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II-restricted T cells. *J.Exp.Med.* **172**, 947-954.

JENNINGS, S.R.,BONNEAU,R.H.,SMITH,P.M.,WOLCOTT,R.M., and CHERVENAK,R. (1991). CD4-positive T lymphocytes are required for the generation of the primary but not secondary CD8-positive cytolytic T lymphocyte response to Herpes Simplex Virus in C57BL/6 mice. *Cell.Immunol.* **133**, 234-252.

JIN, Y.,SHIH,J.W.K. and BERKOWER,I. (1988). Human T cell response to the surface antigen of hepatitis B virus (HBsAg). Endosomal and nonendosomal processing pathways are accessible to both endogenous and exogenous antigen. *J.Exp.Med.* **168**, 293-306.

JOHNSON, D., GHOSH-CHOUDHURY, G., SMILEY, J., FALLIS, L. and GRAHAM, F. (1988). Abundant expression of herpes-simplex virus glycoprotein gB using an adenovirus vector. *Virology*, **164**, 1-14.

JONES, P.D. and ADA, G.L. (1986). Influenza virus antibody secreting cells in the murine lung during a primary influenza virus infection. *J.Virol.* **60**, 614-619.

KAPIKIAN, A.Z.,MITCHELL,R.H.,CHANNOCK,R.M.,SHVEDOFF,R.A. and STWEART,C.E. (1969). An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am.J.Epidemiol.* **89**, 405-421.

KARRE, K.,LJUNGGREN,H.G.,PIONTEK,G., and KIESLING,R. (1986). Selective rejection of H-2 deficient lymphoma varients suggests alternative immune defence strategy. *Nature* **319**, 675-678.

KARUPIAH, G.,WOODHAMS,C.E.,BLANDEN,R.V., and RAMSHAW,I.A. (1991). Immunobiology of infection with recombinant vaccinia virus encoding murine IL-2. *J.Immunol.* **147**, 4327-4332.

KAWAMURA, H. and BERZOFSKY,J.A. (1986). Enhancement of antigenic potency *in vitro* and immunogenicity *in vivo* by coupling the antigen to anti-immunoglobulin. *J.Immunol.* **136**, 58-65.

KAWAMURA, H.,ROSENBERG,S.A. and BERZOFSKY,J.A. (1985). Immunisation with antigen and interleukin-2 in vivo overcomes Ir gene low responsiveness. *J.Exp.Med.* **162**, 381-386.

KIM,H.W.,CANCHOLA,J.G.,BRANDT,C.D.,PYLES,G.,CHANOCK,R.M.,JENSEN, K. and PARROT,R.M. (1969). Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am.J.Epidemiol.* **89**, 422-434.

KLAUS, G.G.B.,PEPYS,M.B.,KITAJIMA,K. and ASKONAS,B.A. (1979). Activation of mouse complement by different classes of mouse antibody. *Immunology* **38**, 687-695.

KLEID, D.G.,YANSURA,D.,SMALL,B.,DOWBENKO, D., MOORE, D.M., GRUBMAN, M.J., MCKERCHER, P.D., MORGAN, D.O., ROBERTSON, B.H. and BACHRACH,H.L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: response in cattle and swine. *Science* **214**, 1125-1129.

KLEIN, J.R. (1986). Ontogeny of the Thy-1, Lyt-2 and murine intestinal intraepithelial lymphocyte-characterisation of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. *J.Exp.Med.* **164**, 309-313.

KLIPSTEIN, F.F., ENGERT, R.F., CLEMENTS, J.D. and HOUGHTAN, R.A. (1983). Vaccine for enterotoxogenic *Escherichia coli* based on synthetic heat-stable toxin cross-linked to the B subunit of heat-labile toxin. *J.Infect.Dis.*, **147**, 318-326.

KNAPP, W.,RIEBER,P.,DORKEN,B.,SCHMIDT,R.E.,STEIN,H. and BORNE,A.E.G. (1989). Towards a better definition of human leucocyte surface molecules. *Immunol.Today* **10**, 253-258.

KNIGHT, S.C. and STAGG,A.J. (1993). Antigen-presenting cell types. *Current Opinion In Immunology* **5**, 374-382.

KONING,F.,STRINGLI,G.,YOKOYAMA,W.M.,YOMODA,H.,MALOY,W.L., TSCHACHLER,E.,SHERACH,E.M.,COLIGAN, J.E. (1987). Identification of a T3-associated gamma delta T cell receptor on Thy-1 and dendritic epidermal cell lines. *Science.* **236**, 834-837

KURT-JONES, E.A.,LIANO,D.,HAYGLASS,K.A.,BENACERRAF,B.,SY,MAN-SUN and ABBAS,A.K. (1988). The role of antigen-presenting B cells in T cell priming *in vivo*. *J.Immunol.* **140**, 3773-3778.

KUSHNER, S.R. (1978). An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids. In *Genetic Engineering*, Edited by. H.B. Boyer and S. Nicosia; Elsvier/North-Holland, Amsterdam, p17.

KUZIEL,W.A.,TAKASHIMA,A.,BONYHADI,M.,BERGSTRESSER,P.R.,ALLISON, J.P.,TIGELAAR,R.E., and TUCKE, A (1987). Regulation of T cell receptor gamma delta chain RNA expression in murine THY-1 and dendritic epidermal cells. *Nature.* **328**, 263-266.

LAGRANDERIE, M.,MURRAY,A.,GICQUEL,B.,LERCLERC,C. and GHEORGHIU,M. (1993). Oral immunization with recombinant BCG induces cellular and humoral immune responses against the foreign antigen. *Vaccine* **11**, 1283-1290.

LASKY, L.A.,GROOPMAN,J.E.,FENNIE,C.W., BENZ, P.M., CAPON, D.J., DOWBENKO, D.J., NAKAMURA, G.R., NUNES, W.M., RENZ, M.E. and BERMAN, P.W. (1986). Neutralisation of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. *Science* **233**, 209-210.

LAYTON,G.T., HARRIS, S.J., GEARING, A.J.H., HILL-PERKINS, M., COLE, J.S., GRIFFITHS, J.C., BURNS, N.R., KINGSMAN, A.J. and ADAMS, S.E. (1993). Induction of HIV-specific cytotoxic T lymphocytes in vivo with hybrid HIV-1 V3:Ty-virus-like particles. *J.Immunol.* **151**, 1097-1107.

LEECE, R. and HIRST,T.R. (1992). Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin in a marine *Vibrio* and in a mutant that is pleiotropically defective in the secretion of extracellular proteins. *J.Clin.Microbiol.* **138**, 719-724.

LEELING, J.L.,MUNI,I.A.,HELMS,R.J. and JOHSON,N. (1979). Rates of release of subcutaneously injected antigens in the rat. *Allergy* **34**, 339-344.

LEGRAIN, P.,GOUD,B. and BUTTIN,G. (1986). Increase in retroviral infection *in vitro* by binding of antiretroviral antibodies. *J.Virol.* **60**, 1141-1144.

LEHNER, T., BERGMEIER, L.A., PANAGIOTIDI, C., TAO, L., BROOKES, R., KLARINSKIS, L.S., GEARING, A.J.H., and ADAMS, S.E. (1992). Induction of mucosal and systemic immunity to a recombinant simian immunodeficiency viral protein. *Science*, **258**, 1365-1369.

LEONARD, C.K.,SPELLMAN,M.W.,RIDDLE,L.,HARRIS,R.J.,THOMAS,J.N. and GREGORY,T.J. (1990). Assignment of intracellular disulphide bonds and characterisation of potential glycosylation sites of the type 1 recombinant human-immunodeficiency-virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J.Biol.Chem.* **256**, 10373-10382.

LETVIN, N.L., DANIEL, M.D., SEHAL, P.K., DeROSIERS, R.C., HUNT, R.D., WALDORN, L.M., MACKOY, J.J. SCHMIDT, O.K., CHALIFOUX, L.V. and KING, N.W. (1985) Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science*, **230**, 71-73.

LEVY, J.A. (1993). Pathogenesis of Human Immunodeficiency Virus Infection. *Microbiol.Rev.* **57**, 183-289.

LIANG, X.,LAMM,M. and NEDRUD,J.G. (1988). Oral administration of cholera toxin sendai virus conjugate potentiates gut and respiratory immunity against sendai virus. *J.Immunol.* **141**, 1495-1501.

LIPSCOMBE, M.,CHARLES,I.G.,ROBERTS,M. and DOUGAN,G. (1991). Intranasal immunization using the B subunit of the *Escheriachi coli* heat-labile toxin fused to an epitope of the *Bordetella pertussis* P.69 antigen. *Mol.Microbiol.* **5**, 1385-1392.

LONG, E.O. and JACOBSON,S. (1989). Pathways of viral antigen processing and presentation to CTL: defined by the mode of virus entry? *Immunol.Today* **10**, 45-48.

LOVGREN, K. (1988). The serum antibody responses distributed in subclasses and isotypes after intranasal and subcutaneous immunisation with influenza virus immunostimulatory complexes. *Scand.J.Immunol.* **27**, 241-245.

LUCKOW, V.A. and SUMMERS,M.D. (1988). Trends in the development of baculovirus expression vectors. *Biotech.* **6**, 47-55.

LUI, Y. and JANEWAY,C.A. (1992). Cells that present both ligand and costimulatory activity are the most efficient inducers of clonal expansion of normal CD4 T cells. *Proc.Natl.Acad.Sci.USA* **89**, 3845-3849.

LYCKE, N. and STROBER,W. (1989). Cholera toxin promotes B cell isotype differentiation. *J.Immunol.* **142**, 3781-3787.

LYCKE, N. and HOLMGREN,J. (1986). Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology* **59**, 301-308.

LYCKE, N.,BROMANDER,A.K.,EKMAN,L.,KARLSSON,U. and HOLMGREN,J (1989). Cellular basis of immunomodulation by cholera toxin *in vitro* with possible association to the adjuvant function *in vivo*. *J.Immunol.* **142**, 20-27.

LYCKE, N.,TSIYI,T. and HOLMGREN,J. (1992). The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. *Eur.J.Immunol.* **22**, 2277-2281.

LYCKE, N. and HOLMGREN,J. (1989). Adoptive transfer of gut mucosal antitoxin memory by isolated B cells 1 year after oral immunisation with cholera toxin. *Infect.Immun.* **57**, 1137-1141.

MACATONIA, S.E.,TAYLOR,P.M.,KNIGHT,S.C. and ASKONAS,B.A. (1989). Primary stimulation by dendritic cells induces antiviral proliferation and cytotoxic T cell responses *in vitro*. *J.Exp.Med.* **169**, 1255-1264.

MACKETT, M.,SMITH,G.L. and MOSS,B. (1982). Vaccinia virus- a selectable eukaryotic cloning and expression vector. *Proc.Natl.Acad.Sci.USA* **79**, 7415-7419.

MALE, D., CHAMPION, B. and COOKE, A. (1987). In *Advanced Immunology*, Gower Medical Publishing, Chapter 2, 2.2-2.13.

MANIATIS.T., FRITSCHE, E.F. and SAMBROOK, J. (1982). *Molecular cloning: A Laboratory Manual.* New York, Cold Spring Harbor Laboratory.

MATSUI,K.,BONIFACE,JJ.,REAY,P.A.,SCHILD,H.,FAZEKAS,D.E.S.T.,GROTH,B. and DAVIS,M.M. (1991). Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* **254**, 1788-1791.

MAZANEC, M.B.,LAMM,M.E.,LYN,D.,POTNER,A. and NEDRUD,J.G. (1992). Comparison of IgA versus IgG monoclonal antibodies for passive immunization of the murine respiratory tract. *Virus Res.* **23**, 7-12.

MBAWUIKE, I.N. and WYDE,P.R. (1993). Induction of $CD8^+$ cytotoxic T cells by immunisation with killed influenza virus and effect of cholera toxin B subunit. *Vaccine* **11**, 1205-1213.

MCALEER, W.J.,BUYNAK,E.B.,MAIGETTER,R.Z.,WAMPLER,D.E.,MILLER,W.J. and HILLEMAN,M.R. (1984). Human hepatitis B vaccine from recombinant yeast. *Nature* **307**, 178-180.

MCBRIDE, B.W. and WARD,K.A. (1987). Herpes simplex-specific IgG subclass response in hepatic-keratitis. *J.Med.Virol.* **21**, 179-189.

McDERMOTT, M.R.,LUCKACHER,A.E.,BRACIALE,V.L.,BRACIALE,T.J. and BIENSTOCK,J. (1987). Characterisation and *in vivo* distribution of influenza-virus-specific T-lymphocytes in the murine repsiratory tract. *Am.Rev.Respir.Dis.* **135**, 247-249.

MCEWEN, J.M.,LEVI,R.,HORWITZ,R.J. and ARNON,R. (1992). Synthetic recombinant vaccine expressing influenza haemaglutinin epitope in Salmonella flagellin leads to partial protection in mice. *Vaccine* **10**, 405-411.

MCGHEE, J.R. and KIYONO,H. (1993). New Perspectives in Vaccine Development: Mucosal Immunity to Infections. In *Infectious Agents and Disease.* pp.1-19.

MCGHEE,J.R.,MESTECKY,J.,DERTZBAUGH,M.T.,ELDRIDGE,J.H.,HIRASAWA,M. and KIYONO,H. (1992). The mucosal immune system: from fundemental concepts to vaccine development. *Vaccine* **10**, 75-88.

MCLACHLAN, D.R.C.,LUKIW,W.J. and KRUCK,T.P.A. (1989). New evidence for an active role of aluminium in Alzheimer's disease. *Can.J.Neurol.Sci.* **16**, 490-497.

MELLOR,J.,MALIM,M.H.,GULL,K.,TUITE,M.F.,MCCREADY,S.,DIBBAYAWAN,T., KINGSMAN,S.M. and KINGSMAN, A.J. (1985). Reverse-transcriptase activity and TY RNA are associated with virus-like particles in yeast. *Nature* **318**, 583-586.

MESTECKY, J. (1987). The common mucosal immune system and current strategies for induction of immune response in external secretions. *J.Clin.Immunol.* **7**, 265-276.

MESTECKY, J. and McGHEE,J.R. (1987). Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. *Adv.Immunol.* **40**, 153-245.

MEUER, S.C.,DUNMANN,H.,MEYER ZUM BUSCHENFELDE,K-H. and KOHLER,H. (1989). Low-dose interleukin-2 induces systemic immune responses against HBsAg in immunodeficient non-responders to hepatitis B vaccination. *Lancet* **i**, 15-17.

MICHALEK, M.T.,BENACERRAF,B. and ROCK,K.L. (1992). The class II MHC-restricted presentation of endogenously synthesised ovalbumin displays clonal variation, requires endosomal/lysosomal processing, and is up-regulated by heat shock. *J.Immunol.* **148**, 1016-1024.

MILICH, D.R.,MCLACHLAN,A.,THORNTON,G.B. and HUGHES,J.L. (1987). Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T-cell site. *Nature* **329**, 547-549.

MIMS, C.A. (1987). Pathogenesis of infectious disease. 3rd edition. Academic Press, London. pp250-269.

MOLDOVEANU,Z.,STASS,J.K.,GILLEY,R.M.,RAY,R.,COMPANS,R.W., ELDRIDGE,J.H., TICE,T.R. and MESTECKY, J. (1989). Immune responses to influenza virus in orally and systemically immunised mice. *Curr.Top.Microbiol.Immunol.* **146**, 91-99.

MOLODOVEANU, Z.,NOVAK,M.,HUANG,W.Q. and GILLEY,R.M. (1993). Oral immunisation with influenza virus in biodegradable microspheres. *J.Infect.Dis.* **167**, 84-90.

MOORE, M.W.,CARBONE,F.R. and BEVAN,M.J. (1988). Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **54**, 777-785.

MOREIN, B. (1988). The ISCOM antigen-presenting system. *Nature* **332**, 287-288.

MORGAN, A.J.,FINERTY,S.,LOVGREN,K.,SCULLION,F.T. and MOREIN,B. (1988). Prevention of Epstein-Barr (EB) virus induced lymphoma in cottontop tamarins by vaccination with the EB virus envelope glycoprotein pg340 incorporated into iscom. *J.Gen.Virol.* **69**, 2093-2096.

MOSMANN, T.R.,CHERWNSKI,H.,BOND,M.W.,GIELDLIN,M.A. and COFFMAN,R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J.Immunol.* **136**, 2248.

MOURITSEN, S.,HANSEN,A.S.,PETERSEN,B.L. and BUUS,S. (1992). pH dependence of the interaction between immunogenic peptides and MHC class II molecules. *J.Immunol.* **148**, 1438-1444.

MUELLER, D.L.,JENKINS,M.K., and SCHWARTZ,R.H. (1989). An accessory cell derived co-stimulatory signal acts independently of protein kinase C to allow T cell proliferation and prevent induction of non-responsiveness. *J.Immunol.* **142**, 2617-2628.

MULLBACKER, A. and BLANDEN,R.V. (1978). Murine cytotoxic T-cell responses to alphavirus is associated with H-2 Dk. *Immunogenetics* **7**, 537.

MURPHY, B.R. (1994). Mucosal Immunity to Viruses. In *Handbook of Mucosal Immunology*, Edited by Ogra, P.L., 333-313.

MURPHY,B.R.,GRAHAM,B.S.,PRINCE,G.A.,WALSH,E.E.,CHANOCK,R.M.,KARZ ON,D.T. and WRIGHT,P.F. (1986). Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. *J.Clin.Microbiol.* **23**, 1009-1014.

MURPHY, B.R.,NELSON,D.L.,WRIGHT,P.F.,TIERNEY,E.L.,PHELAN,M.A. and CHANOCK,R.M. (1982). Secretory and systemic immunological response in children infected with live attenuated influenza A virus vaccines. *Infect.Immun.* **36**, 1102-1108.

NABEL, E.G.,PLAUTZ,G. and NABEL,G.J. (1990). Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. *Science* **249**, 1285-1288.

NATHANSON, N. and LANGMUIR,A.D. (1963). The Cutter incident. Poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955. I. Background. *Am J Hygiene* **78**, 16-28.

NEDRUD, J.G.,LIANG,X.,HAGUE,N. and LAMM,M.E. (1987). Combined oral/nasal immunisation protects mice from Sendai virus infection. *J.Immunol.* **139**, 3484-3492.

NEILL, R.J.IVINS,B.E. and HOLMES,R.K. (1983). Synthesis and secretion of the plasmid-coded heat-lablie enterotoxin of *Escherichia coli* in *Vibrio cholerae*. *Science* **221**, 289-291.

NEUTRA, M.R.,PHILLIPS,T.L.,MAYER,E.L. and FISHKIND,D.J. (1987). Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch. *Cell.Tissue.Rev.* **247**, 537-546.

NGUYEN, T.D.,BOTTREAU,E.,BERNARD,S.,LANTIER,I. and AYNAUD,J.M. (1986). Neutralising secretory IgA and IgG do not inhibit attachment of transmissible gastroenteritis virus. *J.Gen.Virol.* **67**, 939-947.

NICK, S.,HAWS,J.,FRIEBEL,K.,BIRR,C.,HUNSMANN,G. and BAYER,H. (1990). Virus neutralising and enhancing epitopes characterised by synthetic oligopeptides derived from the feline leukaemia virus glycoprotein sequence. *J.Gen.Virol.* **71**, 77-83.

NIXON-GEORGE, A.,MORAN,T.,DIONNE,G.,PENNEY,C.L.,LAFLEUR,D. and BONA,C. (1990). The adjuvant effect of stearyl tyrosine on a recombinant subunit hepatitis B surface antigen. *J.Immunol.* **144**, 4798-4802.

NORMENT, A.M.,SALTER,R.D.,PARHAM,P.,ENGELHARD,V.H. and LITTMAN,D.R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**, 79-81.

NOVOTNY, J.,HANDSCHUMACHER,M. and BRUCCOLERI,R.E. (1987). Protein antigenicity: a static surface property. *Immunol.Today* **8**, 26-31.

NOVOTNY,J.,HANDSCHUMACHER,M.,HABER,E.,BRUCCOLIERI,R.E.,CARLSON, W.B.,FANNING,D.W., SMITH,J. and ROSE, G.D. (1986). Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). *Proc.Natl.Acad.Sci.USA* **83**, 226-230.

NUCHTERN, J.G.,BONIFACINO,J.S.,BIDDISON,W.E. and KLAUSNER,R.D. (1989). Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation. *Nature* **339**, 223-326.

OGRA, P.L.,COPPOLA,P.R.,MACGILLIVRAY,M.H. and DZIERBRA,J.L. (1974). Mechanism of mucosal immunity to viral infections in IgA immunoglobulin-deficiency syndromes. *Proc.Soc.Exp.Biol.Med.* **145**, 811-816.

ORVELL, C. and NORRBY,E. (1977). Immunologic properties of purified Sendai virus glycoproteins. *J.Immunol.* **119**, 1882-1887.

OSTERHAUS, A.D.M.E.,SUNDQUIST,B.MOREIN,B.,VAN STEENIS,G. and UYTDEHAAG,F. (1986). Comparison of an experimental rabies iscom subunit vaccine with

inactivated dog kidney cell vaccine. In: *Proceedings of the First International Veterinary Immunology Symposium*,Guelph,Canada.

OUTLAW, M.C. and DIMMOCK,N.J. (1990). Mechanisms of neutralisation of influenza virus on mouse tracheal epithelial cells by mouse monoclonal polymeric IgA and polyclonal IgM directed against the viral haemagglutinin. *J.Gen.Virol.* **71**, 69-76.

OUTLAW, M.C. and DIMMOCK,N.J. (1991). Insights into neutralisation of animal viruses gained from study of influenza virus. *Epidemiol.Infect.* **106**, 205-220.

OWEN, R.L. (1977). Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* **72**, 440-445.

OWEN, R.L.,PIERCE,N.F.,APPLE,R.T. and CRAY,W.C. (1986). M cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's patches: a mechanism for antigen sampling and for microbial transepithelial migration. *J.Infect.Dis.* **153**, 1108-1112.

OWENS R.L. and JONES, A.L. (1974). Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* **66**, 189-203.

PALVA, E.T., HIRST, T.R., HARDY, S.J.S., HOLMGREN, J. and RANDALL, L. (1981). Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. *J.Bacteriol.* **146**, 325-330.

PANICALI, D. and PAOLETTI,E. (1982). Construction of poxviruses by cloning vectors-insertion of the thymidine kinase gene from herpes-simplex virus into the DNA of infectious vaccinia virus. *Proc.Natl.Acad.Sci.USA* **79**, 4927-4931.

PAPPO, J. and ERMAK,T.H. (1989). Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake. *Clin.Exp.Immunol.* **76**, 144-148.

PIERCE, N and GOWANS, J (1975). Cellular kinetics of the intestinal response to cholera toxin in rats. *J.Exp.Med.* **142**, 1550-1563.

PELLEQUER, J.L.,WESTOF,E. and VAN REGENMORTEL,M.H.V. (1991). Overview of methods for predicting the location of continuous epitopes in proteins from their primary structures. *Methods Enzymol.* **203**, 176-201.

PENNY, C.L.,ETHIER,D.,DIONNE,G.,NIXON-GEORGE,A., ZAGHOUANI,H., MICHON,F., and JENNINGS,H.B (1993). Further studues on the adjuvanticity of stearyl tyrosine and ester analogues. *Vaccine* **11**, 1129-1134.

PERKUS, M.E.,PANICALI,D.,MERCER,S. and PAOLETTI,E. (1986). Insertion and deletion mutants of vaccinia virus. *Virology* **152**, 285-297.

PERKUS, M.E.,PICCINI,A.,LIPINSKAS,B.R. and PAOLETTI,E. (1985). Recombinant vaccinia virus-immunisation against multiple pathogens. *Science* **229**, 981-984.

PETERSON, L.J.,BENSON,W.W. and GRAEBER,F.O. (1955). Vaccination-induced poliomyelitis in Idaho. *JAMA* 159, 241-244.

PFAFFENBACH, G.,LAMM,M.E., and GIGLI,I. (1982). Activation of guinea pig alternative complement pathway by mouse IgG immune complex. *J.Exp.Med.* **155**, 231-247.

POLYDEFKIS,M.,KOENIG,S.,FLEXNER,C.,OBAH,E.,GEBO,K.,CHAKRABARTI,S., EARL,P.L.,MOSS,B. and SILICI (1990). Anchor sequence-dependent endogenous processing of human immunodeficiency virus 1 envelope glycoprotein gp160 for CD4+ T cell recognition. *J.Exp.Med.* **171**, 875-887.

PRINCE, G.A.,HORSWOOD,R.L. and CHANOCK,R.M. (1985). Quantitative aspects of passive immunity to respiratory synctial virus infectionin infant cotton rats. *J.Virol.* **55**, 517-520.

PRINCE,A.,JENSON,A.B.,HEMMING,V.G.,MURPHY,B.R.,WALSH,E.E., HORSWOOD,R.L., and CHANOCK,R.M. (1986). Enhancement of respiratory synytial virus pulmonary pathology in cotton rats by prior intramuscular injection of formalin-inactivated virus. *J.Virol.* **57**, 721-728.

PYLE, S.W.,MOREIN,B.,BESS,J.W.JR.,AKERBLOM,L., NARA,P.L., NIGIDA, S.M., LERCHE, N.W., ROBEY, W.G., FISCHINGER, P.J. and ARTHUR, L.O. (1989). Immune response to immunostimulatory complexes (ISCOMs) prepared from human immunodeficiency virus type 1 (HIV-1) or the HIV-1 external envelope glycoprotein (gp120). *Vaccine* **7**, 465-473.

RADAELLI, A., GIMELLI, M., CREMONESI, C., SCARPINI, C. and DE GIULI MORGHEN, C. (1994a). Humoral and cell-mediated immunity in rabbits immunised with live non-replicating avipox recombinants expressing the HIV-1_{SF2} *env* gene. *Vaccine*, **12**, 1110-1116.

RADAELLI, A. and DE GIULI MORGHEN, C. (1994b). Expression of HIV-1 envelope gene by recombinant avipox viruses. *Vaccine*. **12**, 1101-1109.

RAGER-ZISMAN, B. and ALLISON,A.C. (1973). The role of antibody and host cells in the resistance of mice against infection by Coxsackie B-3 virus. *J.Gen.Virol.* **19**, 329-338.

RAMPHAL, R.,COGLIANO,R.C.,SHANDS,J.W. and SMALL,P.A. (1979). Serum antibody prevents lethal murine influenza pneumonitis but not tracheitis. *Infect.Immun.* **25**, 992-997.

RANDALL, R.E. (1989). Solid matrix-antibody-antigen (SMAA) complexes for constructing multivalent subunit vaccines. *Immunol.Today* **10**, 336-339.

RANDALL, R.E. and RUSSELL,W.C. (1990). Paramyxovirus persistence, consequences for host and virus. In *The Paramyxoviruses*,D.W.Kingsbury, eds.Plenum. pp299-319.

RANDALL, R.E., HANKE,T,YOUNG,D. and SOUTHERN,J.A. (1993). Two-tag purification of recombinant proteins for the construction of solid matrix-antibody-antigen (SMAA) complexes as vaccines. *Vaccine* **11**, 1247-1252.

RANDALL, R.E.,YOUNG,D.,HANKE,T.,SZAWLOWSKI,P. and BOTTING,C. (1994). Purification of antibody-antigen complexes containing recombinant SIV proteins: comparison of antigen and antibody-antigen complexes for immune priming. *Vaccine* **12**, 351-358.

RANDALL, R.E.,YOUNG,D.F.and SOUTHERN,J.A. (1988). Immunisation with solid matrix-antibody-antigen complexes containing surface or internal virus structural proteins protects mice from infection with the paramyxovirus, simian 5. *J.Gen.Virol.* **69**, 2517-2526.

RANDALL, R.E.and YOUNG,D.F. (1988). Humoral and cytotoxic T cell Immune responses to internal and external structural proteins of Simian Virus 5 induced by immunization with solid matrix-antibody-antigen complexes. *J.Gen.Virol.* **69**, 2505-2516.

RANDALL, R.E., YOUNG, D.F., GOSWAMI, K.K.A. and RUSSELL, W.C. (1987). Isolation and characterisation of monoclonal antibodies to simian virus 5 and their use in revealing antigenic differences between human, canine and simian isolates. *J.Gen.Virol.* **68**, 2769-2780.

RAUH, L.W.and SCHMIDT,R. (1965). Measles immunization with killed virus vaccine. *American Journal of Diseases of Children* **109**, 232-237.

REDDY, R.,ZHOU,F.,NAIR,S.,HUANG,L. and ROUSE,B.T. (1992). *In vivo* cytotoxic T lymphocyte induction with soluble proteins administered in liposomes. *J.Immunol.* **148**, 1585-1589.

REDMOND,M.J.,OHMANN,H.B.,HUGHES,H.P.A.,SABARA,M.,FRENCHICK,P.J.,POKU,S.K.A., and IJAK,M.K.(1991). Rotavirus particles function as immunological carriers for the delivery of peptides from infectious agents and endogenous proteins. *Mol.Immunol.* **28**, 269-278.

RELYVELD, E.H. (1986). Preparation and use of calcium phosphate adsorbed vaccines. *Dev.Biol.Stand.* **65**, 131-136.

RENEGAR, K.B. and SMALL,P.A. (1991). Passive transfer of local immunity to influenza virus infection by IgA antibody. *J.Immunol.* **146**, 1972-1978.

RIORDAN, J.R. (1993). The cyctic fibrosis transmembrane conductance regulator. *Annu.Rev.Physiol.* **55**, 609-630.

RODRIGUEZ, D.,RODRIGUEZ,J.R.,RODRIGUEZ,J.F.,TRAUBER,D. and ESTEBAN,M. (1989). Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses. *Proc.Natl.Acad.Sci.USA* **86**, 1287-1291.

ROIZMAN, B. and JENKINS,F.J. (1985). Genetic engineering of novel genomes of large DNA virus recombinants. *Science* **229**, 1208-1214.

ROTHBARD, J.B. (1987). Synthetic peptides as vaccines. *Nature* **330**, 106-107.

RUBIN, D.H.,ANDERSON,A.O. and LUCIS,D. (1983). Potentiation of the secretory IgA response by oral and enteric administration of CP 20, 961. *Ann.N.Y.Acad.Sci.* **409**, 866-870.

SABIN, A.B. and BOUGLER,L.R. (1973). History of Sabin attenuated poliovirus oral live vaccine strains. *J.Biol.Stand.* **1**, 115-118.

SAKANO, H.,HUPPI,K.,HEINRICH,G. and TONEGAWA,S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. *Nature* **280**, 288-294.

SANCHEZ, J. SVENNERHOLM, A.-M., and HOLMGREN, J. (1988). Genetic fusion of non-toxic heat-stable enterotoxin-related decapeptide antigen to cholera toxin subunit B. *FEBS. lett.* **241**, 110-114.

SANDERS, M.E., MAKGOBA, M.W., SHARROW, S., STEPHANY, D., SPRINGER, T.A., YOUNG, H.A. and SHAW, S. (1988). Human memory T lymphocytes express increased levels of three adhesion molecules (LFA-3, CD2 and LFA-1) and three other molecules (UCHL-1, CDw29 and Pgp-1) and gave enhanced IFN-γ production. *J. Immunol.* **140**, 1401-1407.

SANDKVIST, M.,HIRST,T.R. and BAGDASARIAN,M. (1987). Alterations at the carboxyl terminus change assembly and secretion properties of the B subunit of *Escherichia coli* heat-labile enterotoxin. *J.Bacteriol.* **169**, 4570-4576.

SATTERTHWAITE,A.C.,ARRHENIUS,T.,HAGOPIAN,R.A.,ZAVALA,F.,NUSSENZWEIG,V. and LERNER,R.A. (1989). The conformational restriction of synthetic peptides, including malaria peptide, for use as immunogens. *Philosophical Transactions of the Royal Society of London series B* **323**, 565-572.

SCHILLING, J.,CLEVINGER,B.,DAVIE,J.M. and HOOD,L. (1980). Amino acid sequence of the homogenous antibodies to dextran and DNA rearrangments in heavy chain V-region gene segments. *Nature* **283**, 35-40.

SCHLIENGER, K.,MANCINI,M.,RIVIERE,Y.,DORMONT,D.,TIOLLAIS,P. and MICHEL,M-L. (1992). Human immunodeficiency virus type 1 major neutralising determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates. *J.Virol.* **66**, 2570-2576.

SCHODEL, F.,KELLY,S.M.,PETERSON,D.L.,MILICH,D.R. and CURTISS III,R. (1994). Hybrid Hepatitis B virus core-Pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect.Immun.* **62**, 1669-1676.

SCHODEL, F. and WILL, H. (1989). Construction of a plasmid for expression of foreign epitopes as fusion proteins with subunit B of *Escherichia coli* heat-labile enterotoxin. *Infect.Immun.* **57**, 1347-1350.

SCHODEL, F., WILL, H., JOHANSSON, S., SANCHEZ, J. and HOLMGREN, J. (1991). Synthesis in *Vibrio cholerae* and secretion of hepatitis B virus antigens fused to *Escherichia coli* heat-labile enterotoxin subunit B. *Gene*, **99**, 255-259.

SCHULZE-GAHMEN, V.,KLERK,H-D. and BEYREUTHER,K. (1986). Immunogenicity of loop structured short synthetic peptides mimicking the antigen site A of influenza virus haemagglutinin. *Eur.J.Immunol.* **159**, 283-289.

SCHUTZE, M.P.,LERCLERC,C.,JOLIVET,M.,AUDIBERT,F. and CHEDID,L. (1985). Carrier induced epitopic suppression, a major issue for the future of synthetic vaccines. *J.Immunol.* **35**, 2319-2322.

SETTE, A.,SOUTHWOOD,S.,O'SULLIVAN,D.,GAETA,F.C.A.,SIDNEY,J. and GREY,H.J. (1992). Effect of pH on MHC class II-peptide interactions. *J.Immunol.* 148, 844-851.

SHEK, P.N.,YUNG,B.Y.K. and STANACEV,N.Z. (1983). Comparison between multilamellar and unilamellar liposomes in enhancing antibody formation. *Immunology* **49**, 37-44.

SIMPSON, E. and GORDON,R.D. (1977). Responsiveness, to HY antigen Ir gene complementation and target cell specificity. *Imm.Rev.* **35**, 39.

SINGER, S.J. (1992). Intercellular communication and cell-cell adhesion. *Science* **255**, 1671-1677.

SIXMA, T.K., PRONK, S.E., KALK, K.H., WARTNA, E.S., VAN ZANTEN, B.A.M., WITHOLT, B. and HOL, W.G.J. (1991). Crystal structure of a cholera toxin-related heat-labile enterotoxin from *E. coli*. *Nature* **351**, 371-377.

SIXMA, T.K., PRONK, S.E., KALK, K.H., VAN ZANTEN, B.A.M., BERGUIS, A.M. and HOL, W.G.J. (1992). Lactose binding to heat-labile enterotoxin revealed by X-ray crystallography. *Nature* **355**, 561-564.

SIXMA, T.K., KALK, K.H., VAN ZANTEN, B.A.M., DAUTER, Z., KINGMA, J., WITHOLT, B. and HOL, W.G.J. (1993). Refined structure of *Escherichia coli* heat-labile enterotoxin, a close relative of cholera toxin. *J.Mol.Biol.*, **230**, 890-918

SKEA, D.L.,DOUGLAS,A.R.,SKEHEL,J.J. and BARBER,B.H. (1993). The immunotargeting approach to adjuvant-independent immunisation with influenza haemgglutinin. *Vaccine* **11**, 994-1002.

SKEA, D.L. and BARBER,B.H. (1993). Adhesion-mediated enhancement of the adjuvant activity of alum. *Vaccine* **11**, 1018-1026.

SMITH, M.C., FURMAN, T.C., IGNOLIA, T.D. and PIDGEON, C. (1988). Chelating peptide-immobilized metal ion affinity chromatography. A new concept in affinity chromatography for recombinant proteins. *J.Biol.Chem.* **263**, 7211-7215.

SMITH, G.L. and MOSS,B. (1983). Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. *Gene* **25**, 21-28.

SNAPPER, C.M. and PAUL,W.P. (1987). Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* **236**, 944.

SNAPPER, C.M. and MOND, J.J. (1993). Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today.* **14**, 15-17.

SNAPPER, S.B.,LUGOSI,L.,JEKKEL,A.,MELTON,R.E.,KEISER,T.,BLOOM,B.R. and JACOBS,W.R. (1988). Lysogeny and transformation in mycobacteria-stable expression of foreign genes. *Proc.Natl.Acad.Sci.USA.* **85**, 6987-6991.

SNIDER, D.P. and SEGAL,D.M. (1987). Targeted antigen presentation using crosslinked antibody heteroaggregates. *J.Immunol.* **139**, 1609-1616.

SNIDER, D.P. and SEGAL,D.M. (1989). Efficiency of antigen presentation after antigen targeting to surface IgD, IgM, MHC, FcRGRII and B220 molecules on murine splenic B cells. *J.Immunol.* **143**, 59-65.

SNYDER, S.L. and VANNIER,W.E. (1984). Immunologic response to protein immobilized on the surface of liposomes via covalent azobounding. *Biochim.Biophys.Acta* **772**, 288-294.

SOLARI, R.,KUHN,L. and KRAEHENBUHL,J.P. (1985). Antibodies recognising different domains of the polymeric Ig receptor. *J.Biol.Chem.* **260**, 1141-1145.

SOUTHERN, J.A.,YOUNG,D.F.,HEANEY,F.C.,BAUMGARTNER,W. and RANDALL,R.E. (1991). Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *J.Gen.Virol.* **72**, 1551-1557.

SPRINGER, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425-434.

STAHL, S.J. and MURRAY,K. (1989). Immunogenicity of peptide fusions to the Hepatitis B virus core antigen. *Proc.Natl.Acad.Sci.USA* **86**, 6283-6287.

STEPLEWSKI,Z.,SPIRA,G.,BLASZCZYK,M.,LUBECK,M.D.,RADBRUCH,A.,ILLGES,H.,HERLYN,D., and RAJEWSKY,K. (1985). Isolation and characterisation of anti-monosialoganglioside monoclonal antibodies. *Proc.Natl.Acad.Sci.USA* **82**, 8653-8657.

STEVENS, T.L.,BOSSIE,A.,SANDERS,V.M.,FERNADEZ-BOTRAN,R.,COFFMAN,R.L.,MOSSMANN,T.R. and VITETTA, A (1988). Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* **334**, 255-258.

STOCKER, B.A.D.,HOISETH,S.K.and SMITH,B.P. (1983). Aromatic dependent *Salmonella* species as live vaccines in mice and calves. *Dev.Biol.Stand.* **53**, 47-54.

STOCKINGER, B. (1992). Capacity of antigen uptake by B cells, fibroblasts or macrophages determines efficiency of presentation of soluble self antigen (C5) to lymphocyte. *Eur.J.Immunol.* **22**, 1271-1278.

STOTT, E.J. (1994). Towards a vaccine against AIDS- lessons from simian immunodeficiency virus vaccines. *Curr. Top. Microbiol.* **188**, 221-237.

STRIBLING, R.,BRUNETTE,E.,LIGGITT,D.,GAENSLER,K., and DEBS,R. (1992). Aerosol gene delivery *in vivo*. *Proc.Natl.Acad.Sci.USA* **89**, 11277-11281.

STROBER, S. and DILLEY, J. (1973). Maturation of B lymphocytes in the rat. I. Migration pattern, tissue distribution and tumour rate of unprimed and primed B lymphocytes involved in the adoptive anti-DNP-response. *J.Exp.Med.*, **138**, 1331.

STROVER, C.K.,DE LA CRUZ,V.F.,FUERST,T.R.,BURLEIN,J.E., BENSON,L.A.,BENNETT,L.T., and BANSAL,G. (1991). New use of BCG for recombinant vaccines. *Nature* **351**, 456-460.

SVENNERHOLM, A.M. and HOLMGREN, J. (1978). Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside-immunosorbent assay (GM1-ELISA) procedure. *Curr.Microbiol.*, **1**, 19-23.

TAGUCHI,T.,AICHER,W.K.,FUHIHASHI,K.,YAMAOTO,M.,MCGHEE,J.R.,BLUESTONE,J.A. and KIYONO,H. (1991). Novel function of intestinal intraepithelial lymphocytes. Murine CD3+, γ/δTcR+ cells produce IFN-γ and IL-5. *J.Immunol.* **147**, 3736-3744.

TAM, J.P. (1988). Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. *Proc.Natl.Acad.Sci.USA* **85**, 5409-5413.

TAYLOR, P.M.,ESQUIVEL,F. and ASKONAS,B.A. (1990). Murine $CD4^+$ T cell clones vary in function *in vitro* and in influenza infection *in vivo*. *Int.Immunol.* **2**, 323-328.

TEW, J.G., PHIPPS, R.P. and MANDEL, T.E. (1980). The maintenance and regulation of the humoral immune response. Persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol.Rev.* **53**, 175-201.

TEW, J.G., KOSCO, M.H., BURTON., G.F. and SZAKAL, A.K. (1990). Follicular dendritic cells as accessory cells. *Immunol.Rev.*, **117**, 185-211.

TEW, J.G. and MANDEL,T.E. (1979). Prolonged antigen half-life in lymphoid follicles of specifically immunised mice. *Immunology* **37**, 69-76.

TONEGAWA, S. (1983). Somatic generation of antibody diversity. *Nature* **302**, 575-581.

TONEGAWA, S.,BRACK,C.,HOZUMI,N. and SCHULLER,R. (1977). Cloning of an immunoglobulin variable region gene from mouse embryo. *Proc.Natl.Acad.Sci.USA*, **74**, 3518-3522

TOWNSEND,A.,BASTIN,J.,BODMER,H.,BROWNLEE,G.,DAVEY,J.,GOTCH,F.,GOULD,K.,JONES,I., and MCMICHAEL,A.J. (1989). Recognition of influenza virus proteins by cytotoxic T lymphocytes. *Phil.Trans.R.Soc.Lond.* **323**, 527-533.

ULMER,J.B.,DONNELLY,JJ.,PARKER,S.E.,RHODES,G.H.,FLEGNER,P.L.,DWARKI,V.J.,GROMKOWSKI,S.H.,DECK,R.R.,DEWITT,C.M.,FRIEDMAN,A.,HAWE,L.A.LEANDER,K.R.,MARTINEZ,D.,PERRY,H.C.,SHIVER,J.W.,MONTGOMERY,D.L. and LIU,M.A. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745-1749.

UNKELESS, J.C.,FLEIT,H. and MELLMAN,I.S. (1981). Structural aspects and heterogeneity of immunoglobulin Fc receptors. *Adv.Immunol.* **31**, 247-270.

VAJDY, M. and LYCKE,N. (1993). Stimulation of antigen-specific T- and B-cell memory in local as well as systemic lymphoid tissues following oral immunization with cholera toxin adjuvant. *Immunology* **80**, 197-203.

VALENZUELA, P.,MEDINA,A.,RUTTER,W.J.,AMMERER,G. and HALL,B.D. (1982). Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* **298**, 347-350.

VAN REGENMORTEL, M.H.V. (1989a). Structural and functional approaches to the study of protein antigenicity. *Immunol.Today* **10**, 266-272.

VAN REGENMORTEL, M.H.V. (1989b). The concept and operational definition of protein epitopes. *Philosophical Transactions of the Royal Society of London series B* **323**, 1-16.

VIDARD, L.,ROCK,K.L. and BENACERRAF,B. (1992). Heterogeneity in antigen processing by different types of antigen-presenting cells. Effect of cell culture on antigen processing ability. *J.Immunol.* **149**, 1905-1911.

VINEY, J.L. and MACDONALD,T.T. (1992). Lymphokine secretion and proliferation of intraepithelial lymphocytes from murine small intestine. *Immunology* **77**, 19-24.

VON BRUNN, A.,BRAND,M.,REICHHUBER,C.,MORYSWORTMAN,C., DEINHARDT,F. and SCHODEL,F. (1993). Principal neutralising domain of HIV-1 is highly immunogenic when expressed on the surface of heptatis B core particles. *Vaccine* **11**, 817-824.

WAGNER, D.K.,CLEMENTS,M.L.,REIMER,C.B.,SNYDER,M.H.,NELSON,D.L.and MURPHY,B.R. (1987). Analysis of IgG antibody responses after live and inactivated influenza A vaccine indicate that nasal wash IgG is a transudate from serum. *J.Clin.Microbiol.* **25**, 559-562.

WALDMAN,R.H.,STONE,J.,BERGMANN,K.C.,KHAKOO,R.,LAZZELL,V., JACKNOWITZ,A., and WALDMAN,E.R. (1986). Secretory antibody following oral influenza immunisation. *Am.J.Med.Sci.* **292**, 367-371.

WALLS, R.S. (1977). Eosinophil response to alum: involvement of T cells in non-antigen-dependent mechanisms. *Proc.Soc.Exp.Biol.Med.* **156**, 431-435.

WALSH, E.E., HALL, C.B., BRISELLI, M., BRANDRISS, M.W. and SCHLESINGER, J.J. (1987). Immunisation with glycoprotein subunits of respiratory syncytial virus to protect cotton rats against viral infection. *J.Infect.Dis.*, **155**, 1198-1204.

WEBER, S.,TRAUNECKER,A.,OLIVERI,F.,GERHARD,W., and KARJALAINEN,K. (1992). Specific low affinity recognition of major histocompatinility complex plus peptide by soluble T-cell receptor. *Nature* **793**, 795.

WEINBERG, A. and MERIGAN,T.C. (1988). Recombinant interleukin 2 as an adjuvant for vaccine-induced protection. Immunisation of guinea pigs with Herpes simplex virus subunit vaccines. *J.Immunol.* **140**, 294-299.

WELTZIN, R.,LUCIAJANDRIS,P.,MICHETTI,P.,FIELDS,B.N., KRAEHENBUHL,J.P. and NEUTRA,M.R. (1989). Binding and transepithelial transport of immunoglobulins by intestinal M cells: Demonstration using monoclonal IgA antibodies against enteric viral proteins. *J.Cell Biol.* **108**, 1673-1685.

WHANG, Y.,SILBERKLANG,M.,MORGAN,A., MUNSHI, S., LENNY, A.B., ELLIS, R.W. and KIEFF, E. (1987). Expression of the Epstein-Barr virus gp 350/220 gene in rodent and primate cells. *J.Virol.* **61**, 1796-1807.

WILSON, D.A., BAILEY, M., WILLIAMS, N.A. and STOKES, C.R. (1991). The *in vitro* production of cytokines by mucosal lymphocytes immunised by oral administration of keyhole limpet haemocynanin using cholera toxin as an adjuvant. *Eur. J. Immunol.* **21**, 2333-2339.

WOLF,J.L.,RUBIN,D.H.,FINBERG,R.,KAUFMAN,R.S.,SHARPE,A.H.,TRIER,J.S. and FIELDS,B.N (1981). Intestinal M cells: a pathway for entry of reovirus into the host. *Science* **212**, 471-472.

WOLFF, J.A.,MALONE,R.W.,WILLIAMS,P.,CHONG,W.,ACSADI,G.,JANI,A. and FELGNER,P.L. (1990). Direct gene transfer into mouse musclee in vivo. *Science* **247**, 1465-1468.

WOOGEN, S.D.,EALDING,W. and ELSON,C.O. (1987). Inhibition of murine lymphocyte proliferation by the B subunit of CT. *J.Immunol.* **139**, 3764-3370.

WU, J.Y.,NEWTON,S.,JUDD,A.,STOCKER,B. and ROBINSON,W.S. (1989). Expression of immunogenic epitopes of hepatitis B surface antigen with hybrid flagellin proteins by vaccine strain of *Salmonella*. *Proc.Natl.Acad.Sci.USA* **86**, 4726-4730.

YURIN, V.L.,RUDENSKY,A.Y.,MAZEL,S.M. and BLECHMAN,J.M. (1989). Immunoglobulin-specific T-B cell interaction. II. T cell clones recognize the processed form of B cells' own surface immunoglobulin in the context of major histocompatibility complex class II molecule. *Eur.J.Immunol.* **19**, 1685-1691.

ZHAORI, G.,SUN,M.,FADEN,H.S. and OGRA,P.L. (1989). Nasopharyngeal secretory antibody response to poliovirus type 3 virion proteins exhibit different specificities after immunisation with live or inactivated poliovirus vaccines. *J.Infect.Dis.* **159**, 1018-1024.

ZINKERNAGEL,R.M.,ALTHAGE,A.,COOPER,S.,KREEB,G.,KLEIN,P.A.,SEFTON, B., FLAHERTY,L., and STIMPFLING,J (1978). Ir-genes in H-2 regulate generation of anti-viral cytotoxic T cells. Mapping to K & D and dominance of unresponsiveness. *J.Exp.Med*, **148**, 592.

ZINKERNAGEL, R.M. and DOHERTY,P.C. (1979). MHC-restricted cytotoxic T-cells: studies on the biological role of polymorphic transplantation antigens determining T-cell restriction, specificity, function and responsiveness. *Adv.Immunol.* **27**, 51-70.

ZINKERNAGEL, R.M. and WELSH,R.M. (1976). H-2 compatibility requirement for virus-specific T cell-mediated effector functions in vivo. I. Specificity of T cells conferring antiviral protection against lymphocytiv choriomenigitis virus. *J.Immunol.* **117**, 1495-1502.