DEVELOPMENT OF SOLID MATRIX-ANTIBODY-ANTIGEN (SMAA) COMPLEXES AS MULTIVALENT SUBUNIT VACCINES

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A Thesis Submitted for the Degree of PhD
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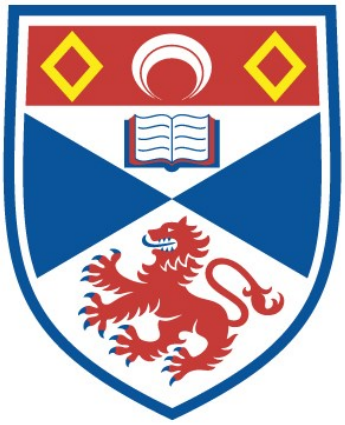

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Development of Solid Matrix-Antibody-Antigen (SMAA) Complexes as Multivalent Subunit Vaccines

by

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DECLARATION

I, Tomas Hanke, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed ..... Date Dec 17, 1993

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ABSTRACT

In the course of the work presented in this thesis, the construction of solid matrix-antibody-antigen (SMAA) complexes as vaccines was further developed. In particular, it was demonstrated that it is feasible to assemble SMAA complexes using a short oligopeptide tag (Pk) attached to the C-termini of antigens and a Pk tag-specific mAb SV5-P-k. In order to facilitate the purification of recombinant proteins for immunization purposes, a second affinity tag was attached to the antigen N-termini. Initially, the N-terminal tag was 26-kDa-large thrombin-removable glutathione *S*-transferase (GST), which permitted first-step purification on immobilized glutathione. However, because of problems with protein insolubility and the proteolytical removal of GST from the hybrid proteins, the GST domain was substituted by an N-terminal 12-amino acid-long tag (His) containing an array of 6 histidines. The His tag was small and thus did not require removal prior to immunization, and allowed purification of His-linked proteins on a nickel-affinity column. Moreover, it was possible to preform nickel-affinity chromatography under protein denaturing conditions, which allowed purification of insoluble or aggregated proteins. In addition, novel prokaryotic expression vectors were constructed for a single-cloning-step addition of these N- and C-terminal tags to proteins of interest. These vectors were used to individually express all non-glycosylated products encoded by the simian immunodeficiency virus (SIV) in *E. coli*. The SIV envelope glycoprotein gp160 with the Pk tag attached to its C-terminus was expressed in insect cells and first-step purified on a lentile lectin column. Following the first purification step on either nickel or lentile columns, all SIV proteins were purified and successfully incorporated into SMAA complexes using anti-Pk tag mAb SV5-P-k. Thus, efficient purification protocols were developed, which purified recombinant proteins via two different affinity tags attached to their N- and C-termini and isolated predominantly full-size proteins. As a stage in achieving the goal of human multivalent vaccines, the SV5-P-k mAb was humanized and is currently being expressed in Chinese hamster ovary cells.

LIST OF ABBREVIATIONS

A	adenine
A#	absorbance at # nm
α-APA	α-anilino-phenylacetamide
ABC	ATP-binding cassette
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
amp	ampicillin
AMV	avian myoblastoma virus
APC	antigen-presenting cells
APS	ammonium persulfate
ATP	adenosine 5'-tris(phosphate)
AZT	3'-azidothymidine, zidovudine, Retrovir
AZTMP	3'-azidothymidine monophospate
BHAP	bis(heteroaryl)piperazine
BIRG-587	11-cyclopropyl-7-methyl-dipyrido-[2,3-b:3',3'-f]1,4-diazepi-6H-5-one
BSA	bovine serum albumin
C	cytosine
CD#	cluster designation
cDNA	complementary DNA
CDR	complenetarity-determining region
cH	chimeric cheavy chain of antibody
C_H#	constant domain of an antibody heavy chain
CMI	cell-mediated immunity
ConA	concanavalin A
cL	chimeric light chain of antibody
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dATP	2'-deoxyadenosine 5'-tris(phosphate)
dCTP	2'-deoxycytidine 5'-tris(phosphate)
ddC	3',2'-dideoxycytidine (also DDC, zalcitabine, HIVID)
ddI	3',2'-dideoxyinosine (also didanosine, Videx)
dGTP	2'-deoxyguanosine 5'-tris(phosphated)
DMAP	4-dimethylaminopyridine
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-tris(phosphates)
d4T	didehydrothymidine (also Stavudine)
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-tris(phosphate)
EDTA	ethylenediaminetetracetic acid
EGTA	ethyleneglycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
env	envelope
ER	endoplasmic reticulum

Fab	antigen-binding fragment of antibody
Fc	crystalizable fragment of antibody
FLT	3'-fluoro-thymidine
G	guanine
gag	group-specific antigen
H-2	designation for the murine MHC locus; chromosome 17
HEL	hen egg-white lysozyme
HLA	human lymphocyte antigen; designation for the human MHC locus; chromosome 6
HIV	human immunodeficiency virus
HLA B27	human lymphocyte antigen (major histocompatibility molecule)
B27	
HPLC	high performance liquid chromatography
ICAM	intercellular cell-adhesion molecule
Ig	immunoglobulin
IPTG	isopropyl-β-*D*-thiogalactopyranoside
kb	kilobases
kbp	kilobase pairs
LB	Luria broth
LCMV	lymphocytic choriomeningitis virus
LFA	lymphocyte function-associated antigen
LMP	low molecular weight polypeptide
LPS	lipopolysaccharide
LTR	long terminal repeat
Mab	monoclonal antibody
MB	multiple-banding antigen
MHC	major histocompatibility complex
MMTV	murine mammary tumor virur
M_r	relative molecular mass
mRNA	messenger RNA
NaAc	sodium acetate
NP	nucleoprotein
NP40	nonidet P-40
nuc	endonuclease
$OD_{\#}$	optical density at # nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque-forming units
pH	pondus hydrogen ($-\log_{10}[H^+]$)

PMEA	9-(2-phosphonomethoxyethyl)-adenin
PMSF	phenylmethylsulphonylfluoride
$prot^i$	inactivated protease
rH	reshaped heavy chain of antibody
rL	reshaped light chain of antibody
RNA	ribonucleic acid
rpm	revolution per minute
RRE	rev-responsive element
rRNA	ribosomal RNA
rt	reverse transcriptase
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SMAA	solid matrix-antibody-antigen complexes
SSC	salt-sodium citrate buffer
ssDNA	single-stranded DNA
strep	streptomycin
T	thymine
TAP	transporter associated with antigen presentation
TAR	sequence at the 5' end of RNA recognized by tat and cellular proteins
TBE	Tris-borate-EDTA buffer
3TC	(-)enantiomer of 2'-deoxy-3'-thiacitidine (also Lamivadine)
TCR	T cell receptor
TE	Tris-EDTA buffer
TEMED	*N,N,N',N'*-tetramethylethylenediamine
tet	tetracycline
THF	thymic humoral factor
TIBO	tetrahydro-imidazo[4,5-jk][1,4]-benzodiazepin-2(1H)-one (also R82913)
TP5	thymopentin
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	transfer RNA
UV	ultra-violet
V_H	variable region of an antibody heavy chain
V_L	variable region of an antibody light chain
v/v	volume per volume ratio
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-b-*D*-galactoside

ABBREVIATIONS FOR AMINO ACIDS

Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
Aspartic acid	asp	D
Cystein	cys	C
Glutamine	gln	Q
Glutamic acid	glu	E
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	val	V

GENETIC CODE

```
TTT phe F TCT ser S TAT tyr Y TGT cys C
TTC phe F TCC ser S TAC tyr Y TGC cys C
TTA leu L TCA ser S TAA OCH Z TGA OPA Z
TTG leu L TCG ser S TAG AMB Z TGG trp W

CTT leu L CCT pro P CAT his H CGT arg R
CTC leu L CCC pro P CAC his H CGC arg R
CTA leu L CCA pro P CAA gln Q CGA arg R
CTG leu L CCG pro P CAG gln Q CGG arg R

ATT ile I ACT thr T AAT asn N AGT ser S
ATC ile I ACC thr T AAC asn N AGC ser S
ATA ile I ACA thr T AAA lys K AGA arg R
ATG met M ACG thr T AAG lys K AGG arg R

GTT val V GCT ala A GAT asp D GGT gly G
GTC val V GCC ala A GAC asp D GGC gly G
GTA val V GCA ala A GAA glu E GGA gly G
GTG val V GCG ala A GAG glu E GGG gly G
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UNITS

°C	degrees Celsius(temperature)
g	gram (mass)
m	metre (length)
mol	mole (quantity)
s	second (time)
Ci	Curie [radioactivity; 3.7×10^{10} s^{-1} (disintegrations per second)]
Da	Dalton (relative molecular mass)
F	Faraday (capacitance)
g	gravitational acceleration (9.81 $m.s^{-2}$)
l	litre (volume; 10^{-3} m^3)
M	molar concentration ($mol.l^{-1}$)
min.	minute (time)
S	Svedberg (sedimentation)
U	unit of enzymatic activity

ORDER PREFIXES

d	deci	10^{-1}	k	kilo	10^3
c	centi	10^{-2}	M	mega	10^6
m	milli	10^{-3}	G	giga	10^9
μ	micro	10^{-6}	T	tera	10^{12}
n	nano	10^{-9}			
p	pico	10^{-12}			
f	femto	10^{-15}			
a	atto	10^{-18}			

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INTRODUCTION

The work presented in this thesis is part of a long term effort to develop a universal strategy for construction of multivalent subunit vaccines, and potentially vaccines against human immunodeficiency virus (HIV). The vaccine, that is being developed in our laboratory, is constructed as solid matrix-antibody-antigen (SMAA) complexes.

Part A of the Introduction presents adaptive immune responses generated to viral infections, i.e. branches of immunity the induction of which is primarily targeted in vaccination. Although antibodies represent important antiviral immune responses, part A mainly focuses on antigen processing and presentation to T cells. This is for several reasons: (i) it is an exciting and currently quickly advancing area of immunology; (ii) these processes are believed, in the majority of instances, to be central to the ability of an individual to mount virus-specific effector functions; furthermore, (iii) evidence seems to emerge, that cell-mediated immune responses in humans and the macaque model of HIV infection and acquired immunodeficiency syndrome (AIDS) correlate with protective immunity against immunodeficiency viruses; and, finally, (iv) antigen presentation is the part of immunity, that is believed to be primarily impaired in patients with AIDS. Part B of the Introduction discusses how the knowledge of these pathways is influencing our views on the control of virus infections and, more specifically, the design of anti-viral vaccines. Part C introduces some molecular aspects of HIV infection and AIDS, in which the HIV genome organization, life cycle, pathogenesis and animal models of infection and disease, focusing predominantly on the simian immunodeficiency virus (SIV) infection of macaques, are discussed. Finally, part D states the objectives of the work.

A. IMMUNE RESPONSES TO VIRAL INFECTIONS.

The nonadaptive defence systems of organisms include external skin and internal circulating cells such as phagocytes or natural killer cells, which serve as a first-line defence against viruses. In vertebrates, there is a second-line protection due to a dual system of humoral and cell-mediated immune responses. These immune responses are generated by a cascade of molecular and cellular associations and interactions. They are adaptive, specific and display a long-term memory. The effectors of humoral responses are antibodies produced by B cells, the binding of which to usually extracellular antigens initiates a variety of elimination responses. Virus-infected cells are more effectively cleared by processes involving T lymphocytes, which are capable of killing cells displaying virus-derived peptides. A description of the generation of antigen-specific cell-mediated immune responses with particular emphasis on viral proteins and a shorter section on humoral responses follow below.

A.1. General description of processing and presentation of viral antigens to T cells.

A.1.1. T cell subpopulations.

T-cells recognize antigens on the surface of antigen-presenting cells (APC) in association with molecules encoded by genes of either the class I or class II major histocompatibility complex (MHC) (Zinkernagel and Doherty, 1975), a phenomenon known as MHC restriction. Cluster designation markers CD4 and CD8 distinguish two major populations of T cells, $CD4^-CD8^+$ and $CD4^+CD8^-$, found in the peripheral blood of all vertebrate species. These phenotypes correlate to some extent with T cell functions. MHC class I-restricted T cells ($CD8^+$) function as cytotoxic T-lymphocytes (CTL), which, upon recognition of MHC-antigen complexes, kill the target antigen-presenting cells, e.g. virally infected cells. MHC class II-restricted T cells ($CD4^+$) play several key roles in the generation of immune responses. By producing soluble

factors termed cytokines, they 'help' to carry out the effector functions of the antigen-specific immune responses, i.e. they 'stimulate' both antigen-activated MHC class I-restricted CTL to kill virus-infected cells and antigen-activated B cells to produce antibodies. They also stimulate cells of the non-specific immune responses such as macrophages, granulocytes or eosinophils. Depending on the local cytokine environment created by cells of the innate immunity early in the response, $CD4^+$ T lymphocytes can differentiate into Th1 or Th2 subsets (reviewed by Scott, 1993). Th1 cells produce predominantly tumour necrosis factor and interferon-γ and are best at activating macrophages. Th2 cells make predominantly interleukins IL-4, IL-5, IL-6 and IL-10 and preferentially activate B cells. Some MHC class II-restricted T cells can function as CTL and as such may have an important role in controlling certain virus infections, for example, those of measles and herpes simplex viruses.

A.1.2. Cellular handling of antigens for presentation to T cells.

Pioneering work on antigen presentation demonstrated that internal proteins (e.g. the nucleoprotein), rather than surface glycoproteins, of influenza virus were the major target antigens for influenza virus-specific class I-restricted CTL (Townsend *et al.*, 1984). This was followed by the observation that only part of the gene for influenza virus nucleoprotein (NP) sufficed to sensitize transfected cells to NP-specific CTL lysis (Townsend *et al.*, 1985). The finding that T cells recognize peptides rather than whole native antigens, as is the case with antibodies, was finally demonstrated by the ability of synthetic peptides to sensitize target cells to CTL (Townsend *et al.*, 1986).

During processing for presentation to T cells, antigens are first denatured and partially degraded into peptide fragments. Some of these peptides associate with MHC molecules and the resulting MHC-peptide complexes are transported to and displayed on the cell surface. There are two distinct intracellular pathways, endogenous and exogenous, used for processing antigens for MHC class I- and class II-restricted presentation, respectively. In MHC class I presentation, cytosolic proteins are degraded by proteases and the resulting peptides enter the lumen of the

endoplasmic reticulum (ER) where they become associated with newly synthesized MHC class I molecules. These complexes are then transported through the Golgi apparatus to the cell surface for presentation to specific MHC class I-restricted T cells. For the MHC class II presentation, antigens enter acidic vesicles (endosomes/lysosomes), usually following their capture by specialized cells (see below), where they undergo partial proteolysis. In this compartment, some of the resulting peptides become associated with MHC class II molecules and are transported as MHC-peptide complexes to the cell surface. The intracellular trafficking of MHC molecules and antigens is described in more detail below and is schematically illustrated in Fig. 1.

The primary interactions between T cell receptors and MHC-peptide complexes (Fig. 2 and below) are essential for the eventual generation of both adaptive immunity and some aspects of innate immunity. The parts of peptides participating in the T cell recognition of antigen-presenting cells are called T cell sites and consist of an epitope and an agretope (Sette *et al.*, 1987). Amino acid residues that physically contact a T cell receptor form an epitope and those binding an MHC molecule are called an agretope. However, T cell sites and T cell epitopes are often used interchangeably.

A.1.3. MHC polymorphism.

There is a number of gene clusters encoding MHC class I (e.g. HLA-A, B and C in humans, and H2-K, D and L in mice) and MHC class II (e.g. HLA-DR, DQ and DP in humans; and H-2 I-A and I-E in mice) molecules. Every individual expresses four to six different class I molecules. Not every peptide generated by proteolysis has affinity for a given MHC molecule (below). As a result, only a selected set of peptides (~1%) will be presented to T cells. It has been estimated that each MHC molecule can present approximately 1000 different peptides on a cell, with each species of MHC molecule presenting a different set of peptides. As will also be discussed latter, MHC molecules are highly polymorphic (different alleles of MHC genes are designated with superscript letters, e.g. H $2K^b$ or H-$2K^d$, or by additional

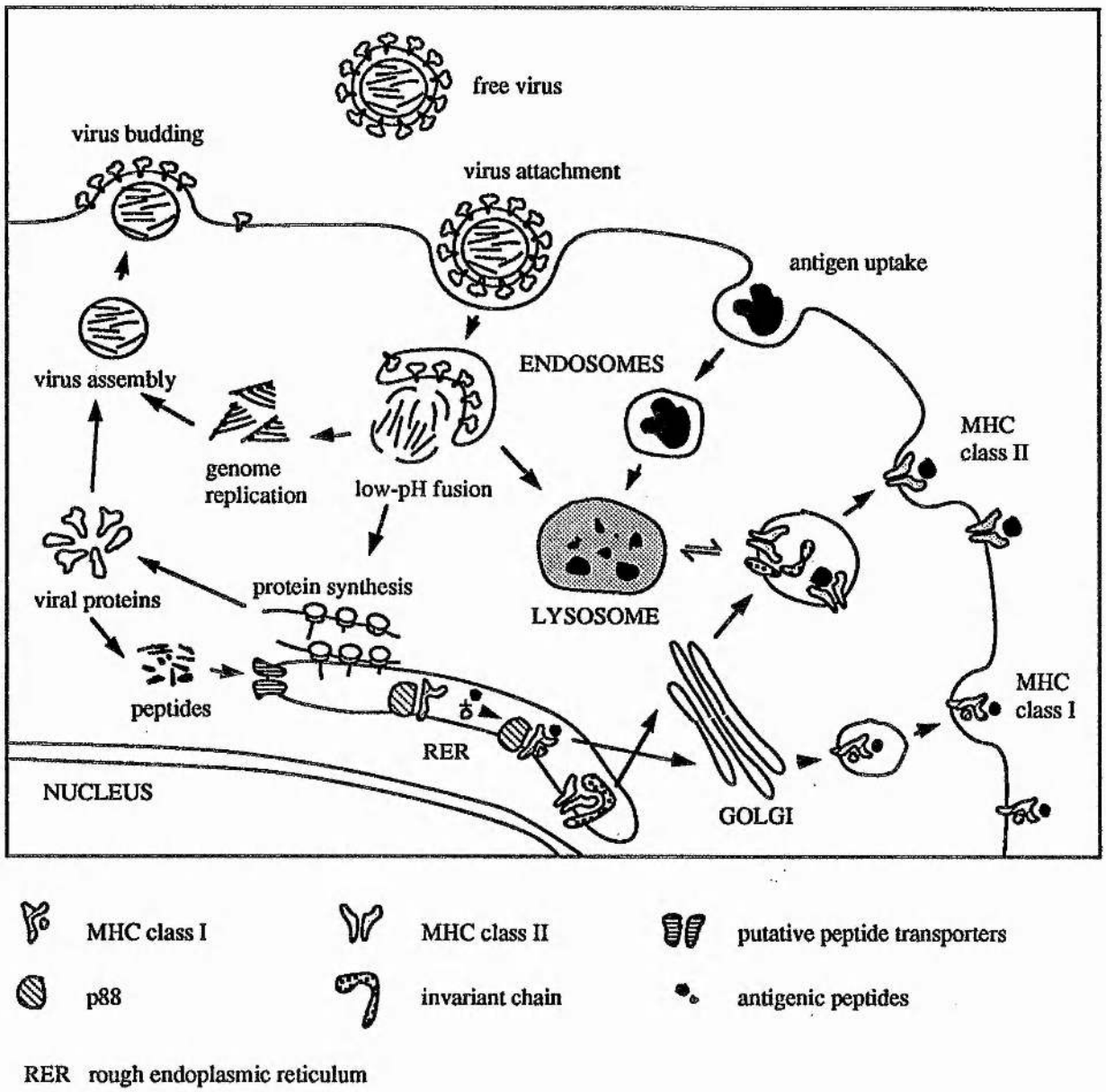


Figure 1. Cellular handling of antigens.
The diagram schematically illustrates the life cycle of a virus (in this case the influenza virus) and how the virus proteins can enter the endogenous (MHC class I) and exogenous (MHC class II) antigen processing and presentation pathways in an antigen-presenting cell. Following partial proteolytic degradation, certain virus-derived peptides associate with either MHC class I or II molecules. The MHC-peptide complexes are then transported to the cell surface where they are specifically recognized by T cells bearing T cell receptors.

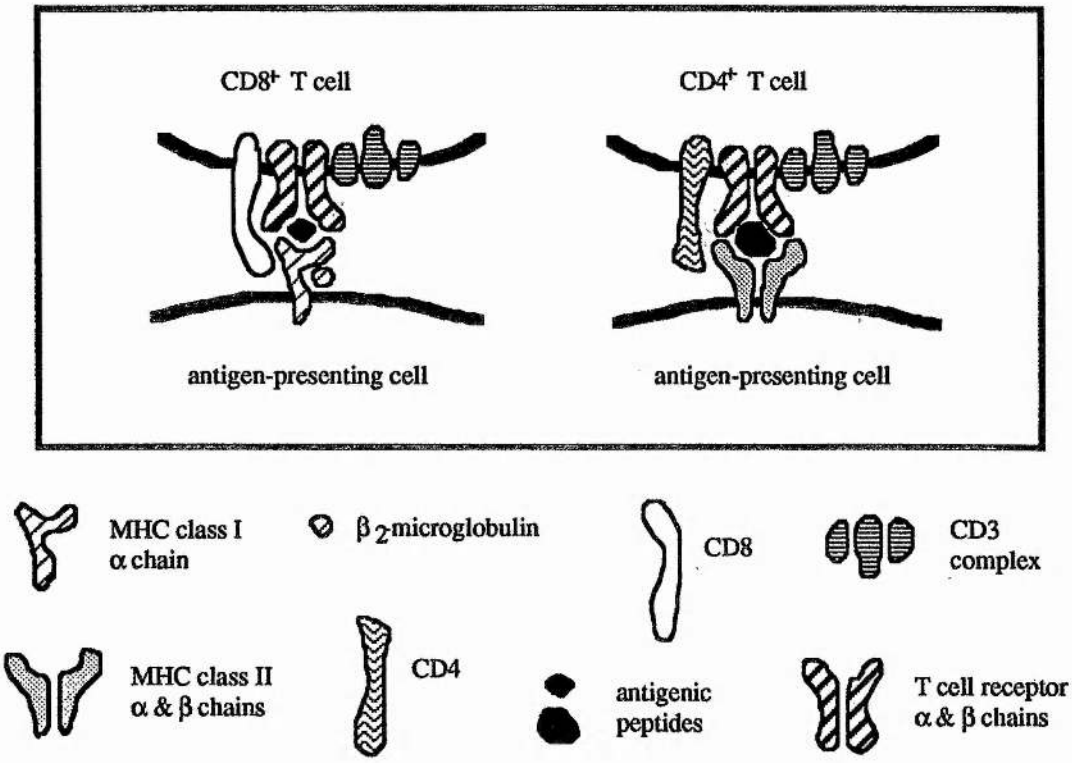


Figure 2. Molecular interactions in antigen-specific T cell recognition of antigen-presenting cells.
The left-hand side of the figure depicts the interactions during the recognition of peptides associated with MHC class I molecules by TCR on a $CD8^+$ T cell (usually a cytotoxic T lymphocyte). The right-hand 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.

numbers and letters, e.g. HLA-Aw68 or HLA-B27) and, as a consequence, different individuals in an outbred population will present different sets of peptides to T cells. If 1000 different peptides do indeed bind to one MHC molecules, then every individual should be able to mount a T cell response to any given virus. Nevertheless, for reasons not clear, it appears that non-responding alleles can be found even for complex viruses (Bennink and Yewdell, 1988).

The MHC polymorphism has arisen in evolution by selection of individuals resistant to different infectious diseases. The extent of MHC polymorphism has been carefully adjusted as a result of two antagonistic processes, the ability to present

peptides vs. positive selection of sufficient variety of T cells in the thymus. An excessive MHC polymorphism would not leave, after negative selection of T cells, broad-enough specificity of the T cell population for the organism defense. The same two antagonistic processes have also determined the length of peptides presented by MHC molecules (below).

A.1.4. Antigen-presenting cells.

Cells, which process antigen for presentation to T cells, are termed antigen-presenting cells (APC). Virtually every nucleated cell expresses MHC class I molecules and thus, when infected, can act as a target for activated CTL. MHC class II molecules are constitutively expressed primarily on haematopoietic cells, but a variety of other cells, e.g. epithelial cells in a number of organs, can express MHC class II molecules when activated by cytokines such as interferon γ.

Although any nucleated cell may act as a target cell for MHC class I-restricted T cells, it appears that the initial activation of resting virgin T cells may require additional factors that can only be supplied by specialized antigen-presenting cells. Thus not every antigenic MHC-peptide complex delivered to the cell surface for presentation to T cells is necessarily immunogenic. This has been demonstrated in several studies of diabetes involving transgenic mice (Ohashi *et al.*, 1991; Oldstone *et al.*, 1991; Schonrich *et al.*, 1991). In one of these studies, mice were made transgenic for lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) so that they expressed the LCMV GP only in pancreatic isle T cells. The mice were phenotypically normal and did not develop diabetes. Neither did the offspring arising from a cross of these mice with other mice that were transgenic for a T cell receptor specific for LCMV GP and thus had a large proportion of T cells that were capable of recognizing MHC-LCMV GP complexes. However, when both the single and double transgenic mice were infected with LCMV, they rapidly developed lethal diabetes (Ohashi *et al.*, 1991). It thus appears that although pancreatic cells can act as target cells for CTL, they are not capable of providing the initial stimuli to activate CTL. However, following virus infection, specialized antigen-presenting cells may activate

a CTL response to LCMV GP resulting in destruction of pancreatic cells and the development of diabetes.

The number of MHC molecules present on the cell surface depends on the cell type and activation state of the cell, which in turn depends on the presence of interferons and other cytokines. It has been estimated that an antigen-presenting cell has to display a minimum of 200 antigenic complexes to be recognized by activated CTL (Christink *et al.*, 1991). The overall avidity of cell-to-cell interactions may be lessened by a negative charge resulting from extensive cell surface glycosylation (Boog *et al.*, 1989). However, in addition to the formation of the MHC-peptide-T cell receptor (TCR) complexes, other auxiliary interactions between cell surface molecules contribute to the intercellular signalling between APC and T cells, e.g. interactions of CD8 and MHC class I or CD4 and MHC class II molecules (Fig. 2), LFA-1 and ICAM-1, LFA-3 and CD2 (for review see Springer, 1990), CD28 and B7, and CD5 and CD72 (Linsley *et al.*, 1991; Vandevelde *et al.*, 1991). Interactions of these molecules increase the overall strength of the T cell binding to APC. These interactions also provide necessary additional signals absent in 'non-professional' APC for the priming of resting T cells, i.e. inducing the progression of T cells from G_0 to G_1 phases of the cell cycle, their proliferation, terminal differentiation and expression of full T cell functions. Because the affinity of TCR for MHC-peptide complexes is very low, the K_D being in the range of 10^{-4}-10^{-5} M (Matsui *et al.*, 1991; Weber *et al.*, 1992), it has been suggested that an antigen-independent adhesion precedes the TCR engagement (Williams and Beyers, 1992).

While any cell infected with virus may act as a target cell for CTL, only specialized antigen-presenting cells may be able to prime CTL. The most potent cell type in this respect seems to be the dendritic cell (Young and Steiman, 1990; Macatonia *et al.*, 1989 and 1991). For example, *in vitro* experiments showed that dendritic cells incubated with T cell site peptides were able to prime CTL responses to influenza virus while similarly treated macrophages or spleen cells failed to do so (Carbone *et al.*, 1988). The special effectiveness of dendritic cells in CTL priming

may be due to their low levels of surface glycosylation (Boog *et al.*, 1989) and high levels of expression of surface adhesion molecules (Freudenthal and Steiman, 1990).

A.2. Antigen presentation by MHC class I molecules.

MHC class I molecules are heterodimers consisting of a transmembrane heavy chain (or α chain) and β_2-microglobulin (Fig. 2). They can present peptides derived from all proteins synthesized in the cell, including those encoded by mitochondria (Loveland *et al.*, 1990; Shawar *et al.*, 1991) as well as proteins targeted or delivered to cytosol by *in vitro* manipulations (Moor *et al.*, 1988). These antigens may include self- and viral proteins or proteins originating from intracellular pathogenic bacteria that enter cytosol (Brunt *et al.*, 1990). Peptides become associated with MHC class I molecules in the ER and/or the Golgi apparatus. The presentation of peptides by MHC class I molecules can be specifically abrogated by treatment of antigen-presenting cells with drug brefeldin A (Misumi *et al.*, 1986). In these cells, brefeldin A blocks the movement of newly synthesized membrane proteins from the endoplasmic reticulum to the Golgi apparatus, while endocytosis and protein synthesis are not inhibited.

A.2.1. Calnexin, an accessory molecule for MHC class I biosynthesis.

When the biogenesis of MHC class I molecules was studied in three murine tumour cell lines, a novel protein of 88 kDa (p88) was identified and partially characterized (Degen and Williams, 1991). Murine p88 and its human counterpart IP90 (Hochstenbach *et al.*, 1992) were later shown to be identical to calnexin, a Ca^{2+}-binding protein present in the ER (Ahluwalia *et al.*, 1992). Calnexin rapidly and quantitatively associates with newly synthesized MHC class I heavy chain and the rate of dissociation of these complexes correlated well with the rate of transport of MHC class I heavy chain from ER to the Golgi. However, calnexin also associates with other nascent proteins in the ER and seems to assist generally with correct protein folding.

Not all newly synthesized MHC class I heavy chains and β_2-microglobulins assemble into stable complexes and excess free β_2-microglobulin is secreted out of the cell (Neefjes and Ploegh, 1988). This suggests that there are limiting amounts of peptides in the ER. Such a situation would ensure that bystanding cells are not sensitized to CTL lysis following capture of secreted antigenic peptides by their unloaded cell surface MHC molecules.

A.2.2. Interaction of peptides with MHC class I molecules and analysis of naturally processed MHC class I-associated peptides.

A landmark in elucidating the way how peptides bind to MHC heterodimers was the determination of crystal structure of human HLA-A2 molecule (Bjorkman *et al.*, 1987). It revealed a single peptide-binding groove on the MHC heavy chain formed by two α-helices and a β-pleated floor. It was only recently, that the atomic details of peptides bound to MHC class I molecules were determined. Three virus-derived synthetic peptides of different lengths and sequences were bound to the murine H-2K^b molecule in an extended β-structure (Fremont *et al.*, 1992; Matsamura *et al.*, 1992; Zhang *et al.*, 1992). The peptides appeared anchored by their termini in the groove with a certain amount of freedom in their central portions. Crystal structures of two human class I molecules, HLA-Aw68 (Silver *et al.*, 1992; Guo *et al.*, 1992) and HLA-B27 (Madden *et al.*, 1992) containing antigenic peptides were also resolved and supported the same general principles for interaction of peptides with class I heavy chain. Peptides bind the groove in the same orientation with their N- and C-termini buried within conserved pockets at the ends of the binding cleft. The pockets hold peptides by bonds made to the peptide main chain of the first two and last two amino acids as well as to the terminal α-amino and carboxyl groups. These interactions are tight, involving a network of van der Waals and hydrogen bonds, and completely exclude solvent molecules from the intermolecular surfaces. Peptide side chains interact with four other pockets in the middle of the binding groove and determine the MHC allele specificity. The middle portion of the peptides

is less constrained by the MHC cleft, which can accommodate longer peptides by bulging away from the binding groove.

These principles may not apply to all MHC class I molecules, or, alternatively, just one end of the antigenic peptide may, in some cases, form sufficiently antigenic complexes. Thus the murine MHC class I H-2L^d molecule was reported to present efficiently two different five-amino acid-long peptides derived from murine cytomegalovirus (Reddehase *et al.*, 1989) and lymphocytic choriomeningitis virus (Whitton *et al.*, 1989). It was shown independently, that the latter peptide, GVYMG, overlapped the most effective nonapeptide RPQASGVYM, and even a four-residue peptide GVYM still, although inefficiently, sensitized target cells to CTL lysis (Schulz *et al.*, 1991).

In order to analyze directly natural peptides bound to MHC molecules, two groups of workers purified murine class I molecules from virally infected cells and acid eluted the bound peptides. Firstly, a vesicular stomatitis virus-derived peptide was isolated that associated with H-2K^b, but did not bind to H-2D^b molecules (Van Bleek and Nathenson, 1990). The second study identified different naturally processed influenza peptides presented by either H-2D^b or H-2K^d molecules (Rotzschke *et al.*, 1990). The eluted peptides contained 8 or 9 residues and their fidelities were confirmed by highly efficient recognition of their corresponding synthetic analogues by CTL.

A.2.3. MHC class I molecules present self-peptides.

The observation that viral peptides stabilize the association of MHC class I heavy chain with β_2-microglobulin and enhance the transport of this complex out of endoplasmic reticulum (Townsend *et al.*, 1989 and 1990) led to the proposal that in uninfected cells, MHC class I molecules are loaded with peptides derived from self-proteins. This notion was in agreement with several X-ray crystallographic studies showing significant electron densities in the binding groove of three human MHC class I molecules, which were hard to resolve, possibly because of the variety of potential self-peptides present in the same protein crystal (Bjorkman *et al.*, 1987;

Garrett *et al.*, 1989; Madden *et al.*, 1991). These speculations were proved correct by eluting pools of heterogeneous self-peptides from H-2K^d, K^b, D^b and HLA-A2.1 molecules (Falk *et al.*, 1991). The majority of these peptides was 8 or 9 amino acids long. The pools of peptides were microsequenced as a mixture and abundances of amino acids at each peptide position revealed agretope motifs characteristic for each MHC molecule. The origins of self-peptides were first identified by separating peptides eluted from HLA-B27 on HPLC and sequencing each peptide individually (Jardetzky *et al.*, 1991). By comparing the peptide sequences with database sequences, 7 out of 11 peptides was possible to identify as being derived from abundant cytosolic or nuclear proteins such as histones, ribosomal proteins and members of the 90K heat-shock protein family. Obviously, mechanisms inducing self-tolerance must have ensured that MHC molecules presenting self-peptides did not induce deleterious immune responses against 'self'. Since then, many allele-specific motifs for MHC class I molecules have been identified (Rammensee *et al.*, 1993).

A.2.4. Prediction of MHC class I T cell sites.

The finding that short synthetic peptides sufficed to sensitize target cells to CTL lysis *in vitro* and could indeed induce specific CTL *in vivo* created high expectations for peptide vaccines and initiated a number of studies identifying immunodominant T cell sites. Several computer algorithms were developed which attempted, with varying degrees of success, to predict T cell sites from the protein amino acid sequences based either on the ability of a particular amino acid stretch to form an amphipathic structure (DeLisi and Berzofsky, 1985) or on the best fit to a consensus sequence common to known T cell sites (Rothbard and Taylor, 1988). With increasing amounts of data, it became apparent that neither approach would be entirely satisfactory. However, from the sequences of the peptides eluted from MHC class I, it was possible to identify MHC allele-specific motifs, i.e. agretope amino acids important for binding to an MHC molecule (Jardetzky *et al.*, 1991; Falk *et al.*, 1991; Hunt *et al.*, 1992; Chicz *et al.*, 1992). Since then, consensus agretope motifs for

a number of MHC molecules have been determined and summarised by Rammensee and colleagues (1993). The MHC class I binding agretope usually consists of one position in the carboxyl-terminus of peptides requiring a certain amino acid side chain, and one position with strong amino acid preferences elsewhere depending on the MHC allele (Maryanski *et al.*, 1989 and 1990; Madden *et al.*, 1991; Fremont *et al.*, 1992). The remaining positions within the peptide are fairly unrestricted. On the other hand, some amino acids at certain positions in peptide ligands might be 'forbidden'.

A.2.5. MHC locus as a cassette for antigen processing and presentation.

MHC class I molecules present peptides derived predominantly from cytoplasmic proteins. Because protein degradation is thought to occur at least initially in the cytoplasm, peptides must cross a membrane to enter the lumen of the ER for association with newly synthesized MHC class I molecules. Genes in the MHC locus code for at least two other classes of proteins that may be involved in antigen processing. These are subunits of a cytoplasmic proteasome thought to be involved in antigen degradation and putative peptide transporters involved in the transport of peptides from the cytosol to the lumen of the endoplasmic reticulum.

A.2.6. Proteolytic degradation of antigens for MHC class I presentation.

It has been suggested that a set of processing-associated genes within the MHC class II locus relates to the proteolytic degradation of antigens for MHC class I presentation (Monaco and McDevitt, 1982, 1984 and 1986). At least two genes in this region, *LMP2* and *LMP7*, code for subunits of a special type of cytoplasmic 20S proteasomes (or multicatalytic protease complexes) called the low molecular weight polypeptide (LMP) complex (Brown *et al.*, 1991). LMP consists of about 13-15 polypeptides encoded by distinct sets of genes. Both of the *LMP2* and *LMP7* genes from MHC have been cloned, but recent evidence showed that these proteasome subunits were not essential for presentation of antigenic peptides from influenza virus and intracellular proteins (Arnold *et al.*, 1992). However, these data did not exclude

a more subtle contribution of the LMP complex to peptide generation. In fact, very little is known about the proteolytic degradation of antigens for class I presentation and alternative mechanisms to proteolysis for generation of peptides have been discussed (Fetten *et al.*, 1991; Boon and Van Pen, 1989).

A.2.7. Transport of peptides from the cytosol to the lumen of the ER and association of peptides with MHC class I molecules.

Studies on mutant cell lines, in which MHC class I heavy chain and β_2-microglobulin were efficiently synthesized, but the MHC heterodimers did not seem to reach the cell surface, have suggested a role for peptide transporters in the translocation of peptides from the cytosol to the lumen of the endoplasmic reticulum. In these cells, the surface expression of MHC molecules was significantly increased by addition of virus-derived peptides, even though the same virus previously failed to sensitize the mutant cells to CTL (Townsend *et al.*, 1989). Further analysis of the phenotypic defect indicated that the presence of peptides was critical for association of the MHC class I heavy chain with β_2-microglobulin (Cerundolo *et al.*, 1990; Ljunggren *et al.*, 1990; Hosken and Bevan, 1990). Some of the cell lines with presentation defect had been previously characterized as having deletions in the class II region of the MHC locus (Townsend *et al.*, 1990; Ljunggren *et al.*, 1990). Two of the missing genes, *TAP-1* and *TAP-2* (for transporter associated with antigen presentation), belonged to the 'ATP-binding cassette' (ABC) superfamily (Monaco *et al.*, 1990; Deverson *et al.*, 1990; Trowsdale *et al.*, 1990). All members of this family contain a domain homologous to a putative ATP-binding site and transport molecules ranging from metal ions and small sugars to proteins, some over 100 kDa, across biological membranes. Reconstitution studies of the mutant cell lines revealed that a defect in either the *TAP-1* or *TAP-2* gene alone resulted in a defect in antigen presentation and a loss of surface expression of MHC class I molecules (Spies and Demars, 1991; Attaya *et al.*, 1992). It has been suggested that the products of the *TAP-1* and *TAP-2* genes form heterodimers that transport peptides across the ER membrane.

Direct evidence for the proposed role for the products of TAP genes the peptide transmembrane transport is still awaited. Relatively convincing functional arguments came from an elegant experiment involving a minigene expressing a T cell site from influenza virus matrix protein introduced into an MHC class I-defective cell line T2 (Anderson *et al.*, 1991). Transfected T2 cells were recognized by class I-restricted CTL only if the matrix T cell site was linked to a signal sequence that targeted the hybrid oligopeptide to the lumen of the ER via the signal-recognition particle-dependent transport mechanism. Thus, if peptides reach the lumen of the ER, T2 cells possess all the machinery required for transporting stable MHC-peptide complexes to the cell surface. Additional support for the role of peptide transporters came from studies which demonstrated that the transporters themselves were polymorphic and could influence the selection of peptides finally displayed on the cell surface by MHC class I molecules (Powis *et al.*, 1992).

On the other hand, several observations question the proposed role for the peptide transporter function of the TAP proteins. Spherical vesicles made from membranes of rough ER called 'rough microsomes' are generally impermeable to proteins lacking the signal sequence. However, short peptides of up to ten residues could enter microsomes generated from the ER of T2 cells (Levy *et al.*, 1991). Since these peptides entered the microsomes in the absence of ATP, it was concluded that ATP-dependent transporters were not involved in this translocation. Although the absence of ATP in preparations is always a disputable point, so far there have been no reports of the binding of nucleoside phosphates to TAP proteins (Gaskins *et al.*, 1992).

There is also some debate as to whether peptides of appropriate length (octapeptides and nonapeptides) for association with MHC class I molecules are generated in the cytoplasm prior to or during the transport into the lumen of the ER, or whether larger peptides are trimmed by proteases in the ER to the appropriate size, possibly after their association with MHC molecules (Wallny *et al.*, 1992). The evidence for trimming of peptides is indirect. It is known that T2 cells can present a limited set of endogenous peptides. Wei and Cresswell (1992) sequenced three such

peptides and found that two, a 9-mer and an 11-mer, were derived from putative signal sequences that would have entered the ER via normal signal-recognition particle-dependent transport. The third peptide of unknown origin was a 13-mer. The presence of correct nonapeptides (in addition to the longer peptides) suggests that some trimming may have occurred in the ER to generate the peptides of the appropriate length (see also Henderson *et al.*, 1992). Specific antigenic peptides can not be detected in cells unless the MHC molecules to which they bind are also present (Falk *et al.*, 1990; Wallny *et al.*, 1992). Thus, if the right-size peptides are generated in the cytoplasm prior to or during the transport into the ER, peptides in the ER unprotected by binding to MHC molecules must have a very short half-life. By the same token, the peptide short half-life in the ER argues for the presence of a strong proteolytic activity in the lumen of the ER.

A.2.8. Non-classical MHC class I molecules.

All the above work has concerned classical MHC class I, i.e. class Ia, molecules. The MHC loci in humans and mice also contain clusters of non-classical class I heavy chain genes designated as MHC class Ib molecules (H-2Q, T and M in mice.) Both class Ia and Ib heavy chains associate with β_2-microglobulin. MHC class Ib molecules display much less polymorphism than class Ia and class II molecules. The allelic differences in the sequences of class Ib genes are only about 1%, which is typical for most other genes and differs markedly from 15% of the class Ia genes. The immunological function of MHC class Ib molecules is largely unknown. Originally, it was suggested that they may simply serve as a sequence pool for generating class Ia polymorphism (Flavell *et al.*, 1986). Evidence for an immune function of MHC class Ib molecules involved several T cell clones bearing $\gamma\delta$ T cell receptors that were restricted by H-2T molecules (Bluestone *et al.*, 1991). Subsequently, it was shown that class Ib-containing MHC complexes could present foreign peptides derived from intracellular bacteria and that H-2M3 molecules specialized in presentation of N-formylated peptides (Loveland *et al.*, 1990; Shawar *et al.*, 1991). The first MHC allele-specific motif was determined for H-2Qa-2

molecules (Rotzschke *et al.*, 1993). The extrapolation of these studies would be that other class Ib molecules can also present foreign peptides, however, no function in combating viral infections has yet been reported.

A.3. Antigen presentation by MHC class II molecules.

MHC class II molecules (HLA-DR, DQ and DP in humans and H-2 I-A and I-E in mice) are heterodimers consisting of transmembrane α and β (heavy) chains (Fig. 2) and, in association with antigenic peptides, stimulate $CD4^+$ T lymphocytes. As outlined above, the pathway of antigen processing for presentation to class II-restricted T cells is clearly different from that for MHC class I presentation. A central role in the MHC class II pathway is played by the acidic vesicles (endosomes/lysosomes) of the cell. It is in these vesicles where the class II antigenic peptides are generated and become associated with MHC class II molecules. The two different processing and presentation pathways reflect the fact that antigens synthesized within the cell (usually derived from the cytosol of an APC) are presented by MHC class I molecules, while any antigen, that reaches the endosomal/lysosomal compartment (usually via receptor mediated endocytosis, phagocytosis or pinocytosis), is processed for MHC class II presentation. In this way, antigens for class II presentation never enter the cytosol and remain inside vesicles within the cell. Processing of antigens for class II presentation is sensitive to lysosomotropic agents such as chloroquine, which buffer the acidic environment of the late endosomal/lysosomal compartment and thus prevent the lowering of internal pH necessary for denaturation and catabolism of internalized antigens.

A.3.1. Binding of peptides to MHC class II molecules.

The molecular details of the MHC class II binding groove-peptide interactions were resolved by determining the crystal structure of peptide-containing HLA-DR1 (Brown *et al.*, 1993). The structure confirmed the validity of the MHC class II structure model based on MHC class I crystallographic data. Differences were

observed in that the ends of the two side α-helices leave the cleft more 'open'. Peptides did not appear to be buried in the groove as in the case of MHC class I molecules, but rather lay straight in the groove projecting from both ends with no apparent kink in the central region. There was a non-polar pocket near one end of the groove in which an anchoring amino acid side chain resided. Interestingly, the crystal structure suggested a dimerization of HLA-DR1 αβ heterodimers.

Peptide-binding assays suggest that class II molecules bind peptides with high affinities reaching 10^{-8} M (Roche *et al.*, 1991) and that strongly binding peptides share allele-specific structural motifs (Jardetzky *et al.*, 1990; O'Sullivan *et al.*, 1991). Site-directed mutagenesis studies of MHC molecules and single-residue substitutions in peptides have provided further information on the importance of individual amino acids in the MHC class II-peptide-TCR interactions (Krieger *et al.*, 1991).

In an analogous series of experiments to the characterisation of naturally occurring peptides bound to MHC class I molecules, peptides have been acid eluted from purified MHC class II molecules and microsequenced. Peptides generated by natural processing and presented by MHC class II complexes were first identified for mouse I-A^b and I-E^b molecules (Rudensky *et al.*, 1991a). They ranged from 13 to at least 17 amino acid residues and this study suggested that the peptides where precisely defined at their N-termini while their C-termini varied. Since then, data has become available on peptides isolated from several other MHC class II molecules (Hunt *et al.*, 1992; Nelson *et al.*, 1992; Kropshofer *et al.*, 1992; Chicz *et al.*, 1992). The size of naturally processed peptides ranged from 12 to 25 amino acids long, depending on the particular class II molecule. In contrast to earlier work, these studies suggest that there seems to be an allele-specific core sequence involved in binding the peptide to the groove of the MHC class II molecule (Hunt *et al.*, 1992; Chicz *et al.*, 1992) and variable length sequences extending at both the N- and C-termini. The 'ragged' ends of peptides argues against the instructive role of MHC class II molecules, i.e. against the trimming of peptides after binding to MHC class II heterodimers.

As with class I molecules, antigenic peptides may contribute to the stability of MHC class II αβ heterodimers (Sadegh-Nasseri and Germain, 1991; Germain and Hendrix, 1991). Two forms of murine class II molecules have been reported in unboiled spleen immunoprecipitates that differed in stability and potentially in conformation. The more stable form remained associated on SDS-PAGE, while the less stable dissociated into α and β monomers. The relative amounts of the two forms varied for different haplotypes and class II molecules. Pre-incubation of spleen cells with antigen (hen egg-white lysozyme; HEL) significantly increased the levels of the stable form and produced a conformational change detectable by a monoclonal antibody. Other studies showed that the stability of class II αβ chain-peptide complexes is also influenced by pH (Jensen, 1990) and the phospholipid composition of membranes harbouring MHC molecules (Roof *et al.*, 1990).

A.3.2. Contact of TCR with MHC-peptide complexes.

Similarly to the antibody variable regions, each α and β chain of TCR contains three hypervariable complementarity-determining regions (CDR) (Davis and Bjorkman, 1988). The conformations of the MHC and TCR complexes suggested that the residues of TCR contacting the peptide epitopes predominantly reside in CDR3, that is in the CDR closest to the constant domain (Chothia *et al.*, 1988; Claverie *et al.*, 1989). The study that supported this model (Jorgensen *et al.*, 1992) involved injecting a series of peptides with a single amino acid substitution in the epitope, i.e. changes affecting the TCR recognition but not the MHC binding, into transgenic mice carrying genes for either the α or β chains of TCR of a T cell line recognizing the original peptide (see Fig. 2). After each immunization of these 'half' transgenic mice, hybridomas were prepared using the responding T cells and genes for their endogenous TCR chains were sequenced. This approach revealed that substitutions in the peptide were reflected by reciprocal substitutions in CDR3 of the 'endogenous' chains.

A.3.3. Prediction of MHC class II T cell sites.

Since the binding of peptides to class II molecules does not appear to be as selective as the binding of peptides to class I MHC molecules, it may prove difficult to predict agretopes that facilitate the binding of peptides to class II molecules. On the other hand, since the binding of peptides to class II molecules appears to be more permissive than the binding of peptides to class I molecules, the parameters used for predicting class II T cells sites may not need to be so rigid.

A.3.4. Proteolytic degradation of antigens for MHC class II presentation and its subcellular localization.

The exact subcellular compartment involved in antigen degradation for MHC class II-restricted presentation continues to generate some dispute. It has been shown for at least some antigens, that unfolding is a prerequisite for efficient proteolysis (Collins *et al.*, 1991; Jensen, 1991). For example, generation of peptides from HEL capable of binding to I-A^k requires the reduction of HEL's four disulphide bonds. This reduction occurred in lysosomal but not in membrane/endosomal fractions (Jensen, 1991). The importance of lysosomes in processing protein antigens was also demonstrated in experiments involving liposomes of various chemical compositions used to deliver four different proteins to either lysosomes or endosomes. In every case, antigens were much more immunogenic when targeted to lysosomes (Harding *et al.*, 1991a and 1991b). Although these results did not exclude endosomal processing for other antigens, lysosomes were the primary catabolic compartment for the tested antigens. In contrast, an ultrastructural study using immunogold labelling of MHC class II demonstrated that endosomes have all the properties required for processing and presentation of antigens (Guagliardi *et al.*, 1990). In essence, there seems to be a late endosomal/lysosomal compartment where degradation of antigens and coupling of the resulting peptides to MHC class II molecules occurs. At present, there is little data available on how MHC class II-peptide complexes are transported out of such a compartment to the cell surface.

Experiments addressing the proteases involved in antigen degradation for MHC class II presentation proved to be largely difficult to interpret, mainly because of the diverse effect of protease inhibitors on APC. The conclusion was that a number of proteases, in particular cathepsin D (Van Noort *et al.*, 1991) and E (Bennett *et al.*, 1992), residing in the endocytic pathway subtly contributed to the proteolysis depending on the cell type and the particular route by which antigens reached the lysosomal compartment. Indeed, the observations that peptides of different lengths and with poorly defined termini can be isolated from MHC class II molecules suggests that they might have been generated by different proteases. This in turn may reflect a multiplicity of routes by which the peptides reached the endosomal/lysosomal compartment. It should also be noted that although MHC class II molecules become loaded with self-peptides (Rudensky *et al.*, 1991a and 1991b; Hunt *et al.*, 1992; Nelson *et al.*, 1992; Kropshofer *et al.*, 1992; Chicz *et al.*, 1992), it appears that a higher proportion of unloaded MHC class II molecules (1-20%) reaches the cell surface compared to MHC class I molecules (<0.3%; Chen and Parham, 1989).

A.3.5. The role of MHC class II-associated invariant chain.

The trafficking of MHC molecules and antigenic peptides within the cell is the key to the differential presentation of peptides by the two classes of MHC molecules. The intracellular pathways and association of MHC class II heterodimers with peptides is in part regulated by a molecular 'chaperon' termed the invariant chain (Teyton *et al.*, 1990). The invariant chain forms a homotrimer and as such temporarily interacts with three MHC class II αβ chain dimers (Roche and Cresswell, 1991a). In doing so, it fulfils two critical functions: it prevents newly synthesized MHC class II molecules from binding antigenic peptides during the early stages of their assembly and transport in the ER (Roche and Cresswell, 1991b), and it directs the assembled MHC class II molecules to the acidic endocytic compartment (see Fig. 1). In this compartment, the invariant chain is proteolytically degraded and releases 'empty' class II αβ dimers which are now available for peptide binding

(Roche and Cresswell, 1991b). Both signals, the first that retains the invariant chain in the ER until its association with the MHC class II molecules and the second responsible for delivering class II-invariant chain complexes into endosomes, are located in the cytoplasmic tail of the invariant chain (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1991).

A.3.6. Presentation of endogenous antigens by MHC class II molecules.

Studies involved in the processing and presentation of the surface antigen of hepatitis B virus (Jin *et al.*, 1988) and antigens of measles and influenza viruses (Jacobsen *et al.*, 1989) demonstrated that MHC class II molecules can present endogenous antigens, although less efficiently than the MHC class I molecules. For this presentation to occur, the endogenous antigens still have to follow the endosomal/lysosomal pathway, for example by internalization of membrane proteins. A series of studies using immunoglobulin λ light chain (Weiss and Bogen, 1989 and 1991) and HEL (Brooks *et al.*, 1991; Moreno *et al.*, 1991) showed that peptides of endogenous origin may also be introduced into the MHC class II pathway by being targeted to particular cellular compartments. Thus peptides derived from mutated λ chains retained in the vacuolar system by preventing the formation of a disulphide bond in the λ variable region, or held in the ER by addition of the KDEL sequence were presented by MHC class II molecules. The same T cell site did not associate with MHC class II molecules when retained in the cytosol or nucleus, or when secreted out of the B cells.

A.4. Viral mechanisms interfering with antigen processing and presentation.

The importance of cell-mediated immunity in controlling virus infections can also be inferred from the different molecular mechanisms viruses have evolved to interfere with the processing and presentation of antigens to T cells. These mechanisms may include a general 'shut off' of host protein synthesis, which is likely

to contribute to the overall decrease of cell surface expression of MHC complexes, as well as more specific mechanisms. To illustrate this point, a few examples of the ways in which adenoviruses, pox viruses and herpesviruses have evolved to evade the immune response will be briefly described.

Striking examples of evasive strategies have been developed by human adenoviruses, which carry in their genomes a cassette of genes protecting infected cells from the immune response. Most of these genes are clustered in an early transcriptional region E3. Functions of all the gene products from this region have not been elucidated, but, for example, glycoprotein 19K binds newly synthesised MHC class I heavy chains and blocks their transport from the ER (Kvist *et al.*, 1978; Paabo *et al.*, 1986; Jefferies and Burgert, 1990), thus preventing the adenovirus-infected cells from displaying MHC class I-peptide complexes (Burgert *et al.*, 1987; Rawle *et al.*, 1989). However, deletion of E3 resulted in disease exacerbation, probably due to increased immunopathology (Ginsberg *et al.*, 1989). Two other proteins from E3 and one from E1B regions protect infected cells against cytolysis induced by tumour necrosis factor. In addition, a protein encoded by E1A region has been demonstrated to down-regulate transcription of the MHC class I gene (Schrier *et al.*, 1983; Bernards *et al.*, 1983).

Different anti-antiviral tactics have been developed by certain poxviruses. Vaccinia virus, for example, possesses genes (B13R and B24R) that code for proteins of the serine-protease inhibitor family (serpins) (Kotwal and Moss, 1989; Smith *et al.*, 1989). A putative function of viral serpins is that they inhibit cellular proteases, some of which are involved in antigen degradation, and thus reduce the presentation of virus-specific peptides. It is known that recombinant vaccinia viruses which express antigens under the control of early promoters can induce CTL responses, while those expressed under the control of late promoters tend only to induce antibody responses (Coupar *et al.*, 1986). Since serpins are expressed late, they may be involved directly in interference with antigen processing and presentation at late times in infection, although direct evidence for their role is still awaited. The production of serpins appears to be only one of a battery of molecules that poxviruses

produce that interfere with the immune response. For example, vaccinia virus also produces soluble homologues to the interleukin-1 (IL-1) and IL-6 receptors (Smith and Chan, 1991; Alcami and Smith, 1992). Since IL-1 and IL-6 have a broad spectrum of effects and are known to play important roles in the regulation of immune responses, it has been suggested that the vaccinia virus homologues can interfere with the immune response by blocking the effects of these cytokines. It has also been suggested that the serpins may interfere with the conversion of pro-IL-1β to the active form, IL-1β (Ray *et al.*, 1992).

Herpesviruses also appear to have developed a number of specific mechanisms for interfering with antigen processing and presentation. Human cytomegalovirus interferes with the transport of MHC class I molecules from the endoplasmic reticulum to the cell surface and, in addition, encodes a protein of high homology with the MHC class I heavy chain capable of binding the human β2-microglobulin (Browne *et al.*, 1990). The proposed function of this protein *in vivo* is to prevent the assembly of MHC class I complexes by sequestering β2-microglobulin, and so render the cytomegalovirus-infected cell unrecognizable by CTL.

A.5. Superantigens.

A separate class of immunogenically potent molecules presented by MHC class II complexes and recognized by T cell receptors is termed 'superantigens' (White *et al.*, 1989). The most distinct feature of superantigens is that a single superantigen can stimulate a large proportion of αβ TCR-bearing T cells, for some reaching up to 30% of the entire T cell population. This hyperimmunogenicity is because the MHC class II-superantigen complexes are recognized only by Vβ (the variable region of β-chain), or in some cases Vγ, of T cell receptor, while MHC class II molecules complexed with conventional antigenic peptides are contacted by all the variable elements of TCR (Vα, Jα, Vβ, Dβ and Jβ). This results in a much higher specificity of T cells recognizing conventional antigens. Indeed, the frequency

of T cell responses to a peptide antigen ranges usually from 10^{-4} to 10^{-6}. The fact that superantigens interact with specific regions of the TCR distinguishes them from polyclonal T cell mitogens, such as concanavalin A. Furthermore, superantigens bind the MHC molecule at sites distinct from the peptide-binding groove (Dellabona *et al.*, 1990) [and different staphylococcal toxins bind to distinct sites on an MHC class II molecule (Scholl *et al.*, 1989)], they do not require intracellular processing (Fraser *et al.*, 1989) and their recognition is only marginally restricted by the MHC allele. Interactions between MHC molecules and superantigens (Dellabona *et al.*, 1990), MHC molecules and Vβ of TCR (Fleischer *et al.*, 1991), and superantigens and Vβ (Pullen *et al.*, 1990; Gascoigne and Ames, 1991) have been demonstrated and shown to be involved in the generation of T cell responses. The sole binding of some superantigens to MHC class II molecules can induce transcriptional activation and increase in MHC-bearing cell adhesiveness (Trede *et al.*, 1991), and may synergize in lymphocyte proliferation (Fuleihan *et al.*, 1991).

A.5.1. Classification and manifestation of superantigens.

Superantigens can be divided into to two categories: foreign and self. The former comprize many bacterial products including e.g. staphylococcal enterotoxins, causative agents of food poisoning and toxic shock syndrome, or streptococcal toxins, whose involvement in multiple autoimmune diseases and immunodeficiencies in humans have been suggested (Heber-Katz and Acha-Orbea, 1989). The latter are exemplified by endogenous antigens found in mice - minor lymphocyte-stimulating determinants (Mls; Festenstein, 1973). There are two major manifestations of self-superantigens. Firstly, certain self-superantigens eliminate during T cell selection in thymus those T cells, which carry TCR Vβ chains specific for these superantigens. Secondly, they cause an extensive proliferation in a mixed-lymphocyte assay, i.e. they are seen as 'alloantigens' in mixed cultures of spleen cells isolated from mouse strains that carry identical MHC genotypes, but differ in their *Mls* loci.

A.5.2. Retroviral superantigens.

Self-superantigens have recently acquired a virological dimension. Although the *Mls* loci are unlinked to MHC, they co-segregate with integrated genomes of endogenous retroviruses, murine mammary tumour viruses (MMTV; Frankel *et al.*, 1991; Woodland *et al.*, 1991; Dyson *et al.*, 1991). In addition, an exogenous MMTV encodes a maternally inherited superantigen (Marrack and Kappler, 1991). The superantigen was transmitted from an infected mother to her offspring after birth (presumably in the milk) and caused a deletion of Vβ14$^+$ T cells in the progeny. Further analysis located the superantigen gene to an open reading frame in the 3' long terminal repeat (LTR ORF) of MMTV (Choi *et al.*, 1991). This finding was confirmed by depletion of Vβ14$^+$ T cells in mice transgenic for both the whole MMTV and for just the LTR ORF. LTR ORF comparison of different MMTV variants located the Vβ specificity to the C-terminal of the gene product (Acha-Orbea *et al.*, 1991). Another and potentially more important finding was a demonstration of a Vβ5-specific superantigen expressed by a defective murine leukaemia virus, which induces an AIDS-like disease in mice (Hugin *et al.*, 1991). B cell lymphomas presenting this retroviral superantigen caused a Vβ5$^+$ T cell proliferation, which was possible to block by *gag* p30-specific antibodies. Thus, superantigens may represent yet another mechanism for perturbation of the fine equilibrium of our immune system, potentially contributing to the development human AIDS.

A.5.3. What is the biological function of superantigens?

What is the function of superantigens that preserved them through the evolution? There are probably 20 to 30 integration sites for MMTV in mice, these are relatively recent in evolution and may not have their counterparts in other mammals as e.g. no endogenous superantigens have yet been detected in humans. In mice, Mls could evolve to delete bacterial toxin-responsive T cells (Marrack *et al.*, 1990). On the other hand, subtle changes in either the *Mls* loci or the respective Vβ regions were found in wild mice, preventing the negative selection of Vβ$^+$ T cells during the maturation in thymus (Cazenave *et al.*, 1990). In addition to negative selection, a role

of Mls in positive selection has been also observed (Benoist *et al.*, 1989) as well as their ability to potentiate conventional antigen presentation (Janeway *et al.*, 1983). This led to the suggestion of a co-ligant function for superantigens (Janeway *et al.*, 1989), whereby Mls-like structures would in special cases stabilize or orient the TCR-MHC interactions. Also, because bacterial superantigen stimulation does not require the CD4 molecule (Fleischer and Schrezenmeier, 1988; Sekaly *et al.*, 1991), these superantigens may substitute for the CD4 function. These properties could be exploited for potentiating immune responses induced by vaccines. In any case, superantigens in mice exert a strong influence on the shaping of the T cell repertoire. Their true biological function and role in autoimmune and immunodeficiency diseases, however, remains obscure.

A.6. Humoral immune responses.

Humoral immune responses defend an organism primarily against the extracellular phase of viral infections. They are mediated by antibodies, which circulate throughout the body in the lymph and in the blood serum. Antibodies are produced by B lymphocytes, which upon recognition of a foreign antigen differentiate into antibody-secreting plasma or memory B cells. For most antigens, these processes are assisted predominantly by helper Th2 cells (above). Once produced, antibodies can bind to a virtually unlimited number of surface structures on the virus and neutralize it by either preventing attachment to and/or penetration into the host cells, or opsonizing the virus for efficient phagocytosis by macrophages. In some instances, these processes may happen more efficiently in the presence of complement.

Antibodies recognize small antigenic determinants called epitopes, which can be continuous or discontinuous (Barlow *et al*, 1986). Discontinuous epitopes depend on the tertiary and quaternary protein structures and are always conformationally sensitive, i.e. are destroyed upon antigen denaturation or proteolytic degradation. Continuous epitopes consist of a linear array of amino acids and are usually conformationally sensitive. Epitopes are defined operationally, that is by recognition

by antigen-binding sites on antibodies, called paratopes. It is assumed that all epitope-paratope interfaces involve a surface area of approximately 700 $Å^2$, as it was shown in the case of lysozyme (Amit *et al.*, 1986). From the known folding patterns of many globular proteins, this assumption implies, that no surface epitope region is likely to contain only a single continuous stretch of amino acid residues. Thus, 'continuous epitope', in fact, usually represents only part of a larger discontinuous determinant. It follows that linear peptide fragments are likely to have only a across-reactive antigenicity with the native protein, from which they were derived. As discussed below, this raises some theoretical questions about peptide vaccines. Several computer programmes have attempted to predict linear cross-reactive fragments from the primary protein structures (Barlow *et al.*, 1986; Blundell *et al.*, 1987). But, as it is with the prediction of T cell sites, these algorithms predicted well some 'continuous' epitopes, while they failed to identify others.

B. CONTROL OF VIRAL DISEASES.

The spread of many viral diseases has been controlled through primary health care and immunoprophylaxis, which involves either active (vaccines) or passive (antisera) immunization. The advances in molecular biology are also resulting in increasingly effective chemotherapies, which in turn put increasing demands on rapid and specific diagnosis.

The severity of virus infection and disease of an individual depends in part on whether the speed of induction of protective immune responses is faster or slower than the virus replication. Among the factors, that may influence the final outcome of virus infection, are the type of virus and the virulence of a particular virus variant, route of infection, previous exposure to the same or similar viruses/antigens, genotype and immune status of an individual, and presence of other compromising factors.

Immunization has been the most reliable means of preventing virus diseases. Predominantly, it is used to protect individuals against the first exposure to pathogens

by increasing the efficiency of protective reaction. While passive immunization supplies virus-specific antibodies, the aim of vaccination is to induce an individual's own natural immunity. This acquired immunity is generated by pre-exposing the immune system to appropriate antigens, which results in a clonal expansion of antigen-specific lymphocytes and generation of circulating antigen-specific memory cells. Most, if not all, of the anti-viral vaccines used to-date prevent viral disease rather than infection. The various types of vaccines and their advantages and disadvantages are discussed below.

B.1. Traditional approaches to virus vaccines.

The conventional method of vaccination utilizes inactivated [e.g. poliomyelitis (Salk), influenza, hepatitis B or rabies] or live-attenuated [e.g. poliomyelitis (Sabin), measles, mumps, rubella or yellow fever] viruses. These approaches are successful, but there are certain concerns or disadvantages associated with their use. Killed virus vaccines require extreme care during the inactivation process to make certain, that no live virus is present and yet that the structure of viral antigens is preserved. The immunity conferred by killed virus vaccines is short and requires additional boosts causing concern about hypersensitivity reactions. In addition, inactivated viruses cannot induce responses to non-structural proteins and the CTL responses are generally poor. Live attenuated vaccines consist of wild type viruses, which are restricted in some critical step in pathogenicity of disease. The necessary considerations with these vaccines are their potential reversion to greater virulence, contamination with agents latently infecting the tissue culture substrates (e.g. simian virus 40, avian leukosis virus or simian cytomegalovirus), establishment of latent infections and limited viability after storage.

B.2. New approaches to viral vaccines.

In certain instances, deleterious rather than beneficial immune responses were primed with the traditional antiviral vaccines. For example, children vaccinated with inactivated measles (Rauh and Schmidt, 1965) or respiratory syncytial (Kim *et al.*, 1976) viruses developed exacerbated diseases after natural infections with these viruses (for review see Randall and Souberbielle, 1990). A great effort is therefore being made to develop alternative vaccination strategies and to improve the efficacy and safety of the existing vaccines. These more or less rational interventions may, however, require more profound understanding of the basis of immune responses to virus infections as well as the biology of virus replication.

B.2.1. Subunit vaccines.

Vaccination with subunit vaccines represents one of the modern antiviral strategies, which involves immunization of individuals with 'harmless' viruses - either chimeric or expressing foreign antigen(s) of interest, purified or more likely recombinant viral antigens, or immunogenic peptides. Unfortunately, small oligopeptides and antigens by themselves are weak immunogens unless they naturally polymerize, as it is in the case of hepatitis B surface antigen (Valenzuela *et al.*, 1982). New formulations and ways of how to increase the efficiency of presentation of poorly immunogenic subunit monomers to the cells of the immune system have been suggested: the use of immunostimulators, such as aluminium gels (Glenny *et al.*, 1926) and muramyl dipeptide (Ellouz *et al.*, 1974), or an assembly of poorly immunogenic monomers into defined multimeric structures, such as the matrix of the adjuvant Quil A of immunostimulating complexes (ISCOMs) (Morein *et al.*, 1984), liposomes (Allison and Gregoriadis, 1974)), solid matrix-antibody-antigen (SMAA) complexes (Randall and Young, 1991), Ty particles (Adams *et al.*, 1987) and many others (reviewed in Gupta *et al.*, 1993). In addition, sustained or timed release of immunogens can mimic booster injections after a single vaccine administration (Tice, 1993).

To induce long lasting protective immunity to many viruses, it may be necessary to induce broad antibody and T cell responses. For the induction of T cell responses, there is a number of problems which have to be considered. Firstly, there are two major pathways for processing antigen for presentation to MHC class I- and class II-restricted T cells. A major problem is how to direct 'non-replicating' antigen to the cytosol of appropriate antigen-presenting cells for the induction of MHC class I-restricted T cells. In some cases involving both viral and non-viral proteins, non-replicating antigens could indeed prime MHC class I-restricted T cells *in vivo*. HIV glycoprotein gp160, influenza haemagglutinin and ovalbumin incorporated into ISCOMs stimulated $CD8^+$ class I-restricted CTL after either subcutaneous injection or oral immunizations (Takahashi *et al.*, 1990; Mowat *et al.*, 1991). Simian virus 5 antigens incorporated into SMAA complexes (Randall and Young, 1991), herpes simplex virus glycoprotein D- and influenza virus-derived peptides attached to a fatty acid as a lipid anchor (Watari *et al.*, 1987; Deres *et al.*, 1989), and soluble ovalbumin (Staerz *et al.*, 1987) have all been reported to induce MHC class I-restricted T cells. The precise mechanisms by which these antigens enter the MHC class I presentation pathway are not known. It remains a possibility that there is a specialized APC that can specifically process exogenous antigen for MHC class I presentation. It has also been suggested that inactivated virus may induce MHC class I-restricted T cells if the inactivated viruses can enter the cell by fusion with the plasma membrane rather than via a low-pH fusion in endocytic vesicles (Bolognesi, 1990). The ability of exogenous antigens to induce MHC class I-restricted CTL is of obvious interest for vaccine design, because safer non-replicating agents would be able to generate immune responses both against virus particles (neutralising antibody) and infected cells (CTL).

B.2.2. Peptide vaccines.

The consequence of MHC polymorphism in terms of cellular immunity is, that different individuals within an outbred population may recognize different T cell-sites on a given protein. Thus, if peptide vaccines are to be developed, it is recognized that

they would have to contain a cocktail of peptides to ensure that a reasonable proportion of individuals would respond to the vaccine. It was estimated, that pools of about six carefully selected CTL sites derived from a particular pathogen could be reactive in nearly the whole population (Mills *et al.*, 1989). For practical purposes, prediction of T cell sites from the amino acid sequences of protein antigens will most likely have to depend solely on the identification of MHC allele-specific agretopes. This approach may be accurate enough to give useful information about potential T cell sites on a protein for possible peptide vaccine development providing the MHC phenotype of a target population has been determined. However, the relevance of these T cell sites to the host immune defence also requires further consideration. The presence of an agretope motif in the amino acid sequence of an antigen does not guarantee that this peptide will associate with MHC molecules, form an antigenic complex and induce a T cell response. The patterns of T cell responses may be influenced by a number of factors other than MHC molecules, including proteins involved in antigen processing (proteasomes and peptide transporters), other immune response genes and also by other background genes (Gammon *et al.*, 1987; Vidovic and Matzinger, 1988).

For similar reasons, identifying peptides for incorporation into potential vaccines by screening for their ability to induce T cell response is also not completely satisfactory. Immunogenic peptides may not be relevant in immune protection for a variety of reasons. The corresponding peptides may not be efficiently generated and/or presented by the natural processing of the native proteins by APC. Thus, after vaccination, peptide-induced T cells specific for such 'cryptic' T cell sites would be irrelevant, because these T cells may never 'find' the MHC-peptide complexes, which they are specific for, on cells infected during the course of natural infection. For example, CTL generated *in vitro* by priming with ovalbumin fragments were unable to recognise target cells transformed with the whole ovalbumin gene (Carbone *et al.*, 1988). In a more complicated experiment, HLA-A2-restricted CTL specific for residues 58-68 of influenza virus A matrix protein were shown to cross-react with the same peptide associated with HLA-Aw69. Since HLA-Aw69 is normally a non-

responding allele, this observation suggests that although the HLA-Aw69 molecule can bind the peptide, it does not present the peptide for other reasons during natural influenza virus infections (Bodmer *et al.*, 1989). Thus, the processing machinery of APC limits the number of T cell sites presented and in turn restricts the complexity of naturally induced T cell responses.

In this respect, there are studies clearly demonstrating a strong bias of immune responses towards particular immunodominant sites over other 'accessible' T cell sites of the same or other viral proteins (Allan and Doherty, 1985). Moreover, 'silencing' the response to a previously immunodominant T cell site can cause a shift of the immune response to the next T cell site in the hierarchy. The mechanisms underlying these immunodominance hierarchies are not yet clearly understood.

Immunization of experimental animals with synthetic peptides, e.g. peptides derived from foot-and-mouth disease (Bittle *et al,* 1982), poliomyelitis (Emini *et al.*, 1983) or herpes simplex (Cohen *et al,*. 1984) viruses, elicited in a number of instances virus-neutralizing antibodies. Despite these encouraging results, design of peptides for the induction of virus-neutralizing antibodies requires much more research to be undertaken. The first limitation of this approach comes from the belief, that most of the antibody epitopes on viral proteins are discontinuous (Barlow *et al.*, 1986; Van Regenmortel, 1989) and cannot be mimicked by a single peptide composed of an array of unmodified amino acids residues. Thus, only 'continuous epitopes' can be easily incorporated into peptide vaccines, which are bound to induce cross-reacting antibodies (see above). Secondly, even linear epitopes are conformational. Although flexibility enables peptides to be more antigenic, it may decrease the epitope immunogenicity, as good immunogenic structures may have to be rigid in order to allow for affinity maturation of antibodies. In one study, looping of a 30-amino acid peptide by a disulphide bond increased the peptide immunogenicity compared to its linear form (Leonard *et al.*, 1990), presumably by generating a tertiary structure and/or restricting the side-chain mobility. Finally, antibody epitopes in peptide vaccines may have to be linked to a T helper cell site, because majority of B cells are dependent on the T cell 'help'.

B.2.3. DNA immunization.

A novel immunization technique is being developed, in which DNA constructs are directly introduced into mammalian tissue *in vivo* (Nabel *et al.*, 1990; Wolff *et al.*, 1990). This gene inoculation can be regarded as a form of subunit vaccination and has generated great hopes for both vaccinology (Wang *et al.*, 1993) and gene therapy (Wilson, 1993). From the vaccine point of view, injection of a normal functional gene mimics features of vaccination with live attenuated viruses. This was shown to induce both neutralizing antibodies to HIV-1 envelope and HIV envelope-specific proliferative responses (Wang *et al.*, 1993). Some of the areas that need further investigation are the efficiency of DNA uptake by cells and the possibility to target the DNA to particular antigen-presenting cells. Examples of the approaches used to stimulate the plasmid DNA uptake are a bupivacaine pretreatment of the injected muscle (Thomason and Booth, 1990), receptor-mediated endocytosis of transferrin-polylysine-DNA conjugates into hematopoietic cells (Zenke *et al.*, 1990) and construction of liposome-DNA complexes (Hyde *et al.*, 1993) or adenovirus-polylysine-DNA conjugates for introducing genes into airway epithelium (Gao *et al.*, 1993a). Obviously, there is a lot to be learned about gene inoculation before this technology becomes generally acceptable for human vaccination.

B.2.4. A case for multivalent vaccines.

For the formulation of subunit vaccines, the cellular handling of antigens has several important consequences which argue for the incorporation of multiple antigens. Firstly, any viral protein, including surface, internal or non-structural proteins, may contain important T cell sites. Thus, multiple viral antigens may have to be included in an effective anti-viral vaccine. Secondly, several antigens may have to be included in a vaccine in order to induce balanced immune responses, as the induction of only appropriate combination of humoral and cell-mediated responses may be desirable to avoid deleterious consequences of vaccination itself and/or exacerbated pathology after subsequent infection with the virus. Thirdly, several factors discussed above, including polymorphism of the proteins involved in the

processing and presentation of antigens, limit the number of T cell sites that can be presented by a single individual, again arguing that several viral antigens may have to be used for vaccination to protect all or most individuals in a target population. The final problem for vaccine design is the antigenic variation of viruses. During the course of infection, viruses are under strong selective pressure imposed upon them by the host immune responses. Thus to cover the most common or even predicted escape mutants, efficient prophylactic and therapeutic vaccines may have to include more then one variant of the same viral antigen.

B.3. Solid matrix-antibody-antigen (SMAA) complexes as multivalent subunit vaccines.

Construction of solid matrix-antibody-antigen (SMAA) complexes has been suggested to be a means of producing effective antiviral vaccines (Randall, 1989). Multispecific immunization with SMAA complexes can be achieved either by incorporating different virus antigens into the same SMAA complex using a mixture of mAbs (Randall and Young, 1989) or by making monospecific SMAA complexes and mixing them prior to injection (Fig. 3).

B.3.1. Immunogenicity of SMAA complexes.

SMAA complexes are highly immunogenic inducing both vigorous humoral and cell mediated immune responses (Randall and Young, 1988). The immunogenic nature of SMAA complexes results in part from the fact that antigens are presented to the cells of the immune system in a stable, particulate and repetitive form. The immune response to SMAA complexes may be also enhanced by the presence of antigen-bound antibodies, which may facilitate the uptake of antigens by APCs via Fc or complement receptors.

Immunization of mice with SMAA complexes that contained either internal or external structural proteins of the paramyxovirus, simian virus 5 (SV5), enhanced the speed of clearance of virus from immunized mice (Randall *et al.*, 1988). The effector

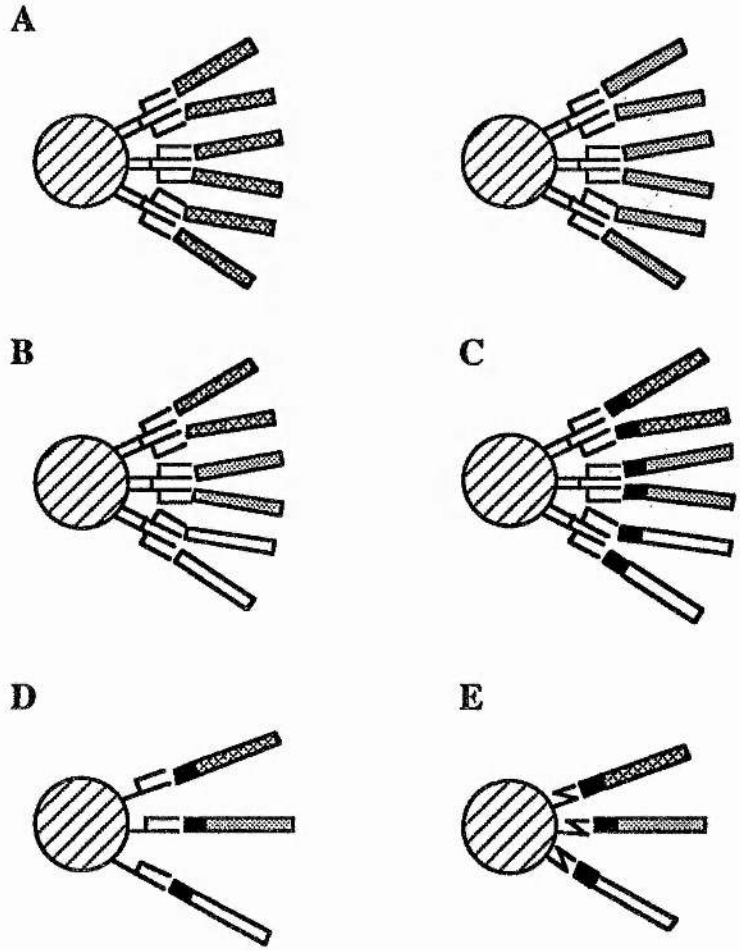


Figure 3. SMAA complexes as multivalent vaccines.
A - Different mAbs are attached to independent solid matrices prior to the binding of antigens. The resulting SMAA complexes may be combined together before being used as immunogens. B - Solid matrix is saturated with a mixture of mAbs prior to the addition of antigens. C - A single tag-specific mAb and tag-linked antigens are used for the construction of SMAA complexes. D and E - The same as C, only Fab of single chain Fv fragments of antibodies, respectively, are employed. Also, additional mAb to host cell antigens and/or immunostimulating proteins, such as cholera toxin subunit B, may be included to enhance particular type of immune responses (not shown). Modified from Randall (1989).

cells primarily responsible for virus clearance were shown to be $CD8^+$ lymphocytes. This together with the demonstration, that SMAA complexes induced class I-restricted cytotoxic T cells (as measured *in vitro*) suggested, that the effector cells responsible for the clearance of virus *in vivo* were indeed $CD8^+$ cytotoxic T cells (Randall and Young, 1991). This set of experiments demonstrated that antigens introduced on SMAA complexes, i.e. non-infectious exogenous agents, can enter intracellular processing pathways leading to an induction of MHC class I-restricted T lymphocytes, although this induction of T cells was less efficient compared to the infectious virus.

B.3.2. Construction of SMAA complexes.

B.3.2.1. Solid matrix.

a) 'Fixed' and killed S. aureus *cells.*

Initially and mainly for practical purposes, preparations of heat-killed and formalin-hardened *Staphylococcus aureus* Cowan A cells (Kessler, 1975) were used as a solid matrix. *S. aureus* cells have a consistently high cell surface density of protein A, which is covalently linked to the peptidoglycan layer of the cell wall (Sjoquist *et al.*, 1972), is stable over a wide range of pH, is quite resistant to denaturing agents and heat (Sjoholm, 1975) and has at least three, but probably no more than four, highly homologous Fc-binding regions (Sjodahl, 1976).

For the construction of SMAA complexes, particulate solid matrices are saturated with specific monoclonal antibodies (mAb), which in turn are saturated with their respective antigens. Practically this means that, in the first step, mAb are concentrated and purified from either ascitic fluids or tissue culture supernatants, and the solid matrix-antibody complexes are used in the next step to purify antigens from crude cellular lysates. The resulting SMAA complexes are used as immunogens.

b) Alum gels.

Immunization of animals with alum-antigen complexes is a well established method for inducing humoral immunity (Klaus and Humphrey, 1977). In this technique, the purified antigen is non-specifically absorbed onto preformed alum

precipitates (aluminium phosphate or hydroxide) or is trapped into the gel during precipitation, a method first described more then 60 years ago (Glenny *et al.*, 1926) It has been shown in our laboratory, that incorporation of antibodies into these alum precipitates, i.e. production of alum-antibody-antigen complexes, further increases the immunogenicity of antigens compared to those of just alum-antigen complexes (Randall *et al.*, 1993b). Thus, the binding of protein to alum via an antibody linkage may increase the efficiency of binding to alum, preserve the protein's native structure, and the antibody may help to trigger certain immunological processes that will enhance the immune responses to alum SMAA complexes. In addition, alum is non-immunogenic and the use of alum gels have been licensed for use in man.

B.3.2.2. Antibodies.

To date we have only used mouse mAb in the construction of SMAA complexes. It is unlikely that it will be possible to use mouse mAb for producing vaccines for general use in humans. SMAA complexes used as human vaccines will have to include human antibodies in order to i) minimize immune responses directed against the antibody component and ii) improve the possible interactions with the human immune system and effector cells. Humoral responses against the murine determinants of the antibody component of the SMAA complexes could significantly reduce the half-life of SMAA complexes in human body, hamper any subsequent immunizations with SMAA complexes, interfere with diagnostic tests involving murine mAb or induce hypersensitivity reactions. The human hybridoma technology has been a considerable technical challenge compounded by immunizing humans and even the best procedures are time-consuming and unreliable, and derived hybridoma may be unstable. Here, recombinant DNA techniques offer a solution. It is possible to construct hybrid antibodies by substituting the genetic information for antigen-binding regions of human heavy and light chains with their rodent counterparts of desired antigen specificity (Fig. 4; Winter and Milstein, 1991). The whole humanized antibody can then be produced in suitable eukaryotic cells (Reichmann *et al.*, 1988; Verhoeyen *et al.*, 1988; Tempest *et al.*, 1991; Page and Sydenham, 1991). However, if the Fc portion of mAb were dispensable for efficient uptake and presentation of

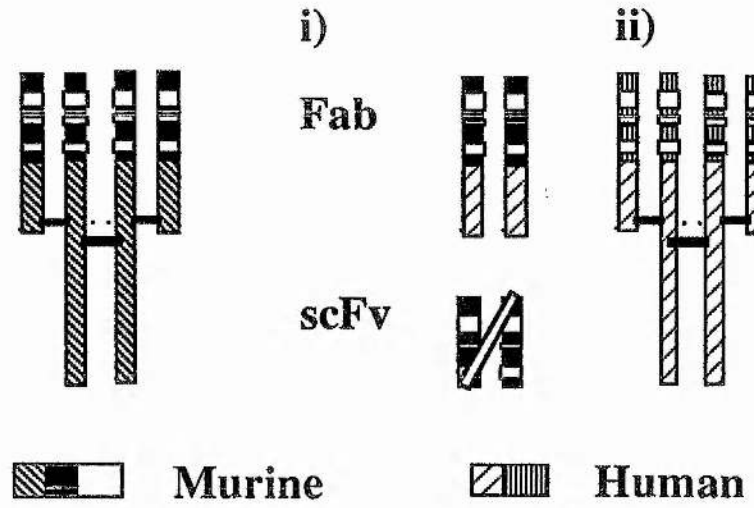


Figure 4. Engineering of antibodies.
i) Antigen-specific heavy and light chain variable regions of a rodent mAb can be expressed in *E. coli* as Fab or Fv fragments of antibodies, the former having human-derived constant regions. ii) Rodent mAb can be completely reshaped by grafting only the antigen-binding complementarity-determining regions onto a human framework. Modified from Winter and Milstein (1991).

antigens by antigen-presenting cells, Fab or Fv fragments could substitute for the whole antibody in SMAA complexes (Fig. 3). Incorporation of Fab or Fv fragments into SMAA complexes would have the advantages of a reduced chance of adverse immune reactions and the availability of bacterial expression systems (Boulot *et al.*, 1990; Skerra *et al.*, 1991).

B.3.2.3. Purification of antigens.

Ideally, successful subunit vaccines will contain recombinant proteins rather than antigens from infected cell lysates, thereby eliminating health risks during both the production and use of the vaccine. Unfortunately, there are no straightforward recipes to follow for protein purification, as each protein has unique physico-chemical properties. Many procedures have been developed over the years and used in various combinations, on one the hand to overcome protein insolubility, proteolytic degradation or aggregation, and on the other to preserve protein structure. Efficiency is yet another important aspect of purification protocols, which is frequently

compromised for purity. Generally, the more purification steps involved, the cleaner is the final product, but the lower the yield. Recombinant DNA technology has again been instrumental in opening new ways of protein purification. Thus, fusion of an affinity tag to one end of the protein of interest may facilitate protein purification and increase protein stability. The tag-based purification procedures may exploit a variety of specific interactions, e.g. between antigenic oligopeptide tags and tag-specific antibodies, enzymes or specific enzyme domains and their substrates, ligands and their receptors, metal-binding amino acid residues and chelated metal ions (reviewed in Sassenfeld, 1990).

C. HIV INFECTION AND AIDS.

The global endemic of human immunodeficiency virus (HIV) continues to grow. According to the World Health Organization statistics, there are 14 million people infected with HIV and 2.5 million have developed acquired immunodeficiency syndrome (AIDS). If the HIV infection continues to spread at the present rate, 30 - 40 million people world-wide will have been infected with HIV by the year 2000. It is likely that most of these people will eventually die of AIDS. At present, the only safe way for altering course and scale of this human disaster is, undoubtedly, in educational campaigns inducing people to modify their high-risk behaviour. Practically, the best hope is in mass vaccination programmes.

C.1. HIV classification.

Human immunodeficiency virus (HIV) is a member of the family of *Retroviridae* (the retroviruses), the genus of *Lentivirinae*. HIV is a small RNA virus, whose virions are of about 80 - 130 nm in diameter and consist of a lipid envelope surrounding an icosahedral nucleocapsid. Currently, there are two well-characterized subtypes of the virus, HIV-1 and HIV-2, both of which cause disease (Clavel *et al.*, 1987).

C.2. HIV genome organization.

HIV genome is composed of two identical copies of a positive-sense single-stranded RNA. The RNA molecules are non-covalently bound and their size is approximately 9,300 nucleotides. The HIV genome consists of 3 large genes encoding polyproteins essential, but not sufficient, for virus replication (see also Fig. 6). These are the *gag* gene coding for the structural proteins of the virion, the *pol* gene, which encodes the various enzymes involved in the processes of reverse transcription, integration of proviral DNA into host chromosomes and virion maturation, and the *env* gene, which encodes glycoproteins present on the virion surface responsible for binding to and penetration into the appropriate target cells. In addition to these 3 genes, HIV carries at least six other genes coding for auxiliary viral proteins nef, rev, tat, vif, vpr and vpu known to play critical roles in both the regulation of HIV gene expression and in morphogenesis and release of infectious virus particles (reviewed e.g. in Haseltine, 1991)

C.3. HIV life cycle.

The replication cycle of HIV begins by virus entry into the target cell. Once inside the cell, the virus genomic RNA is converted into double-stranded DNA that integrates into the host cell genome as a proviral DNA. HIV then 'hijacks' the pre-existing cellular machinery of transcription and translation for production of viral genomes and proteins, which assemble into new nucleocapsid particles. These particles acquire a phospholipid envelope containing viral glycoproteins during egress out of the cell, which results in a release of new fully-infectious virions.

C.3.1. Virus entry.

The initial events of virus infection are the virus binding and penetration of target cells. The virus attachment is facilitated by a high-affinity interaction between the outer portion of virus glycoprotein, gp120, and cellular receptor CD4 (Stein *et al.*,

1987), a surface glycoprotein found on a variety of cells of hematopoietic origin (Dalgleish *et al.*, 1984). However, the gp120/CD4 interaction may not be necessary (Clapham *et al.*, 1989; McKeating *et al.*, 1990) nor sufficient (Tersmette *et al.*, 1989a; Hovanessian *et al.*, 1993) for efficient virus entry. The potential requirement for the cleavage of the third hypervariable domain (the V3 loop) in gp120 lead to an indication, that there may be a requirement for an essential surface co-receptor with proteolytic activity. Hovanessian and colleagues (1993) recently demonstrated, that anti-CD26 mAb or inhibition of its peptidase activity by peptides blocked 80-90% of HIV entry into target cells. Following attachment (and cleavage of the V3 loop), entry of HIV occurs by fusion of the virus phospholipid envelope with the plasma membrane of the cell. Fusion is mediated by the transmembrane portion of the virus glycoprotein, gp41, (Lifson *et al.*, 1986) and is pH independent (Stein *et al.*, 1987).

C.3.2. Synthesis of HIV nucleic acid and provirus integration.

After release of the HIV genome into the cytoplasm, the single-stranded RNA is converted into a double-stranded DNA intermediate. These steps are carried out by virally encoded RNA-dependent DNA polymerase (reverse transcriptase) and ribonuclease H, enzymes characteristic for the retrovirus family, which are delivered to the cytoplasm together with the genome by the infectious particle (Varmus and Swanstrom, 1985). The poor fidelity of reverse transcriptase (Meyerhans *et al.*, 1989) is one of the major problems in the battle with HIV, because it provides a means of escaping both antiviral chemotherapies and immune responses. The double-stranded DNA form of the virus genetic information, now flanked by long terminal repeats (LTR) at both ends, is inserted into the host genomic DNA by another virus-specified enzyme integrase, which is also packed within the virus particle (Farnet and Haseltine, 1990). Once integrated, the proviral DNA becomes permanently associated with the host chromosomes and is vertically transmitted during subsequent cell divisions.

C.3.3. Expression of HIV genes.

Expression of HIV genes is a complex process controlled by both host and viral factors. The production of viral proteins begins with the synthesis of a complete RNA copy of the proviral DNA. Transcription is initiated from the 3'-end of the 5' long terminal repeat and its rate is largely controlled by cellular proteins. The HIV genome contains 4 spice-donor and 6 known splice-acceptor sites resulting in more than 30 distinct processed mRNA species found in infected cells, among which almost every viral gene is positioned as the first open reading frame (ORF) (Schwartz *et al.*, 1990). The mRNA molecules can be either unspliced, or singly or doubly spliced. The exceptions are the *pol* gene, which is translated as a result of a ribosomal frame-shift during translation of the *gag-pol* mRNA (Jacks *et al.*, 1988), resulting in a gag-pol polyprotein, and the *env* gene, which appears to be translated from a bi-cistronic mRNA that also encodes the *vpu* gene product (Schwartz *et al.*, 1990). The gag-pol polyprotein precursor is proteolytically cleaved into functional products by the virus protease.

C.3.4. Nucleocapsid assembly and release of infectious particles.

The capsid precursor protein and the capsid precursor of the replicative enzymes co-assemble at the inner surface of the cell membrane. These proteins have myristic acid attached to their N-termini and insert themselves into the cell membrane (Veronese *et al.*, 1988). The capsid precursor protein contains two copies of a viral RNA-binding motif, so called cysteine-histidine box (Marie and Spahr, 1986). This cysteine-histidine box specifically recognizes the Ψ packaging signal located at the 5'-end of the genomic RNA and ensures, that the RNA is incorporated in the resulting closed spherical particles (Lever *et al.*, 1989). Assembled nucleocapsids bud through the cell membrane, which contains the already digested virus glycoprotein gp41/gp120. Late maturation events include the proteolytic cleavage of capsid protein and replicative enzyme precursors (Oroszlan and Luftig, 1990).

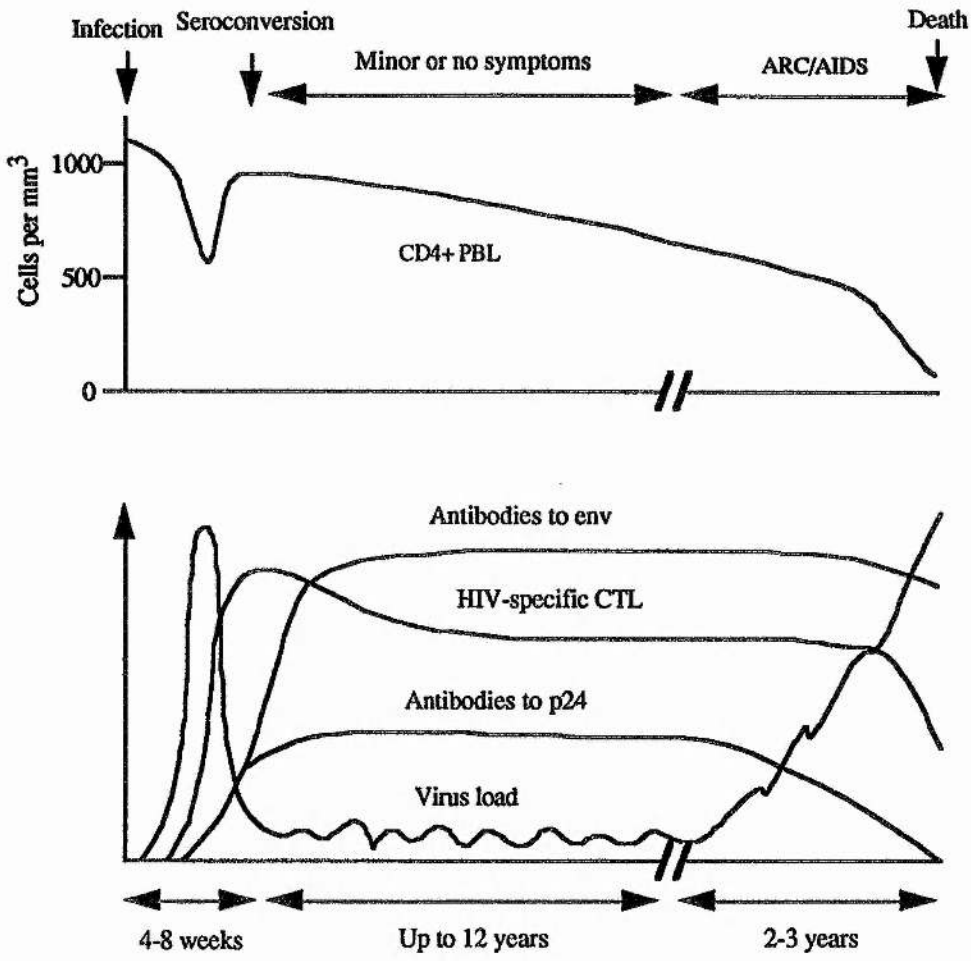


Figure 5. Schematic course of HIV infection and AIDS.

C.4. HIV infection and disease.

HIV is a sexual and blood-born pathogen, the infection of which can have a number of diverse manifestations ranging from subclinical abnormalities to opportunistic infections and malignacies that define the acquired immunodeficiency syndrome (AIDS), the end-stage disease (reviewed by Levy, 1993). The course of HIV infection is schematically depicted in Fig. 5. From the very first description, AIDS has been associated with the depletion of $CD4^+$ T-lymphocytes in the peripheral blood (Gottlieb *et al.*, 1981). However, the $CD4^+$ T helper cells are not the only cells infected, as HIV has been also demonstrated to infect other cells such as

monocytes, macrophages and certain glial cells of the central nervous system. The mechanisms of how HIV causes the immunodeficiency are not entirely clear (Weiss, 1993), but they are driven by a persistent HIV infection (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). There is an increasing body of evidence suggesting, that the decrease in the $CD4^+$ lymphocyte due to viral infection does not solely account for the collapse of the immune system seen in AIDS patients. The central problem seems to be in defective antigen presentation and inappropriate signalling by antigen-presenting cells resulting in T cell anergy and priming cells for activation-induced death (Stanley and Fauci, 1993; Meyaard *et al.*, 1993). One purely theoretical scenario for the immune system defeat follows. In brief: (i) dormant HIV proviruses integrated in the host chromosomes are activated in stimulated lymphocytes during other infections (Tong-Starksen *et al.*, 1989) (ii) the HIV envelope by mimicking allogeneic structures (Wahren *et al.*, 1989; Habeshaw *et al.*, 1992) and/or HIV-associated superantigen (Soudeyns *et al.*, 1993) non-specifically activate lymphocytes and render them susceptible to apoptosis because of the absence of a correct co-signal (Meyaard *et al.*, 1992; Ameisen and Capron, 1991); (iii) antigen-presenting cells produce aberrant cytokine signals, that may affect the overall homeostasis of T cell populations, e.g. by relative dominance of Th2 over Th1 cell subsets (Stanley and Fauci, 1993); (iv) HIV probably kills memory cells faster than they are replenished, which may be one of the consequence of the above malfunctioning of antigen-presenting cells (Helbert *et al.*, 1993); (v) during persistent replication in the lymph nodes, HIV develops sequential escape mutants that are one step ahead of the immunosurveillance; (vi) the accumulation of new HIV antigenic variants eventually overcomes the immune system; and (vii) the fast HIV evolution results in emergence of virulent cytopathic variants (Tersmette *et al.*, 1989b).

C.5. Strategies for the control of HIV infection.

C.5.1. An ideal HIV vaccine.

An ideal anti-HIV vaccine is (i) antigenicly appropriate, i.e. induces protection against different subtypes and variants of HIV. It (ii) induces mucosal and systemic immunities to maximize the likelyhood of protection, and this protection is achieved by (iii) an optimal balance between humoral and cell-mediated immune responses. The protection the vaccine induces is (iv) long-lasting and (v) achieved by a minimal number of doses. The vaccine is (vi) heat stable and (vii) easy to administer, i.e. orally. Finally, the vaccine, that is to have a chance to successfully combat the world-wide HIV epidemic, has to reach the target population, that is to be (viii) affordable for everybody.

C.5.2. Problems with HIV vaccine development.

There is a number of difficulties specific to HIV in the rational development of effective vaccines. (i) Due to the low fidelity of reverse transcriptase, HIV has an ability to rapidly change its genomic sequence. In this way, mutants escaping both immune and drug attacks emerge. (ii) The likely vehicle of transmission is an infected cell rather than a cell-free virus. (iii) HIV can spread directly from cell-to-cell without the need to release an infectious particle. After integration into the host chromosome, HIV can (iv) establish a latent infection, i.e. no expression of viral proteins, and (v) be vertically carried from cell-to-cell as an integrated provirus. (vi) HIV infects the very immune cells upon which the action of vaccine depends. The last point is not a problem for prophylactic vaccines.

After more then a decade of intense effort, there is no cure for AIDS nor is there an effective vaccine against HIV. Before such a vaccine is developed, a number of key questions must be answered. The problem number one is to determine the correlates of human protection, i.e. what are the specific immune responses vaccines have to evoke in order to protect individuals from HIV infection and/or disease. It needs to be established which HIV antigens should constitute the basis of a vaccine,

how the virus antigenic variability can be overcome and how the antigens should be presented to the immune system. Furthermore, reliable surrogate markers for the disease progression need to be identified. Finally, more accurate knowledge is required of how HIV is transmitted and what causes the immune system to collapse.

C.5.3. Non-vaccine strategies for controlling HIV replication.

Vaccination is only one of the possible approaches to controlling the HIV infection and replication. Any step in HIV life cycle and any HIV-derived protein or nucleic acid involved in the virus replication are potential targets for antiviral therapy. The candidate 'hot-spots' for an anti-HIV intervention and some of the corresponding drugs are shown in Table 1. The most favourite target of anti-HIV drugs is the reverse transcriptase. However, to overcome or avoid resistance resulting from the fast generation of escape mutants, a successful chemotherapy may have to be a combination of drugs inhibiting several steps in virus replication at once. No therapeutics targeting DNA synthesis of the second strand, transport of the viral dsDNA from the cytoplasm to the nucleus and integration of the dsDNA into host chromosomes have yet been suggested.

C.6. Animal models of HIV infection and AIDS.

Several animal models have been developed in order to provide a scientific basis for designing vaccination and chemotherapeutic trials in man. These model systems are very helpful for studying both the pathoaetiology of diseases caused by virus infections and for the evaluation of novel anti-viral drugs and potential vaccines. They may provide insights into both the humoral response (including the production of virus-neutralizing antibodies) and the induction of T cell proliferative and MHC-restricted cytotoxic T cell responses.

Table 1. Targeted steps in HIV life cycle and some of the corresponding interfering agents.

Attachment	•sCD4, (Daar *et al.*, 1990) • CD4-Ig (Capon *et al.*, 1989) •sCD4-PE40 [*Pseudomonas aeruginosa* exotoxin A (Ashorn *et al.*, 1990)] •CD4-peptides, dextran sulphate, mAbs
Uncoating	•Hypericin
Reverse transcription	Nucleotide analogues: •AZT* [zidovudine, Retrovir (Mitsuya *et al*, 1985)] •ddC* (DDC, zalcitabine, HIVID) •ddI* (didanosine, Videx) •d4T [Stavudine (Gao *et al.*, 1993b)] •FLT [3'-halo-dideoxypyridine (Matthes *et al.*, 1988)] •PMEA [an acyclic adenine (Balzarini *et al.*, 1989)] •3TC [Lamivadine (Coates *et al.*, 1992)] Non-nucleotide inhibitors: •BHAPs [U-87201E, Atevirdine Mesylate, ATV (Romero *et al.*, 1991)] •U-90,152 (Dueweke *et al.*, 1993) •R-89439 [α-APA derivative (Colebunders *et al.*, 1992)] •L-697, 661 (Saari *et al.*, 1991) •TIBO [R82913 (Pauwels *et al.*, 1990)] •BIRG-587 [nevirapine (Merluzzi *et al.*, 1990)]
RNAseH degradation of template	•AZTMP (Tan *et al.*, 1991)
Viral transcription	•Ro 24-7429 [tat inhibitor (Hsu *et al.*, 1993)] •TAR decoys (Sullenger *et al.*, 1991a)
RNA splicing and transport	•RRE decoys (Sullenger *et al.*, 1991b) •transdominant rev
Protein synthesis	•Antisense mRNA (Izant and Weintraub, 1985)
RNA stability	•Ribozymes (Hasseloff and Gerlach, 1988)
Protein glycosylation	•N-butyl DNJ
RNA packaging and virion assembly	•Myristic acid analogues (Heuckeroth *et al.*, 1990) •transdominant gag, antisense/ribozyme, protease inhibitors
Virus budding	•Interferon α (Pitha *et al.*, 1979; Ho *et al.*, 1985)
Maturation	•A-77003 [protease inhibitor (Erickson *et al.*, 1990)] •Ro 31-8959 [protease inhibitor (Roberts *et al.*, 1990)]
Other	Immunomodulators: •Trental [pentoxifylline, inhibits TNF (Fazely *et al.*, 1991)] •IL-2 [increase in CD4+ and proliferative responses (Schwartz *et al.*, 1991)] •THF and TP5 [augments CMI (Kouttab *et al.*, 1992)]

See Abbreviations for the chemical names of individual drugs; * Approved for human therapy

C.6.1. Search for an animal model.

Since HIV was isolated from patients with AIDS (Popovic *et al.*, 1984), there has been an intensive search for suitable animal models of AIDS. So far the most useful model has proved to be the simian immunodeficiency virus (SIV) infection of several varieties of macaques (reviewed in Schultz and Hu, 1993). A lot of attention has been also focused on the feline immunodeficiency virus (FIV) infection of cats. Although it is possible to grow HIV-1 in mice with severe combined immunodeficiency (SCID) following their reconstitution with human lymphocytes and in chimpanzees, these models are of a limited use because an AIDS-like disease is not produced and the host animals are either rare or difficult to maintain. The relatively recent finding by Agy and colleagues (1992), that pig-tailed macaques can be infected with HIV-1, even though the infection is also non-pathogenic, may provide a better system to test anti-HIV vaccines.

A great amount of attention in HIV vaccinology has been focused on the envelope glycoprotein and its hypervariable V3 loop. However, incorporation of the whole *env* products into vaccines may be somewhat controversial. In addition to the antigenic variability, the sole presence of gp160 or its parts in organism has been implicated in several pathogenic mechanisms potentially contributing to the development of AIDS. These include sensitizing of uninfected $CD4^+$ cells to cytotoxic T lymphocyte lysis (Siliciano *et al.*, 1988), induction of $CD4^+$ cell fusion (Lifson *et al.*, 1986), immunosuppression (Weinhold *et al.*, 1989; Manca *et al.*, 1990), immunological tolerance (Haynes *et al.*, 1993) and apoptosis (Ameisen and Capron, 1991; Szawlowski *et al.*, 1993). Moreover, HIV-1 gp120 expression in astrocytes of transgenic mice resulted in brain neuropathological lesions characteristic of AIDS dementia (Oldstone, 1993).

C.6.2. Studies on simian immunodeficiency virus vaccines.

In 1985, a lentivirus, SIVmac, was isolated and shown to induce AIDS in rhesus macaques (Letvin *et al.*, 1985). Subsequently, other strains of SIV were

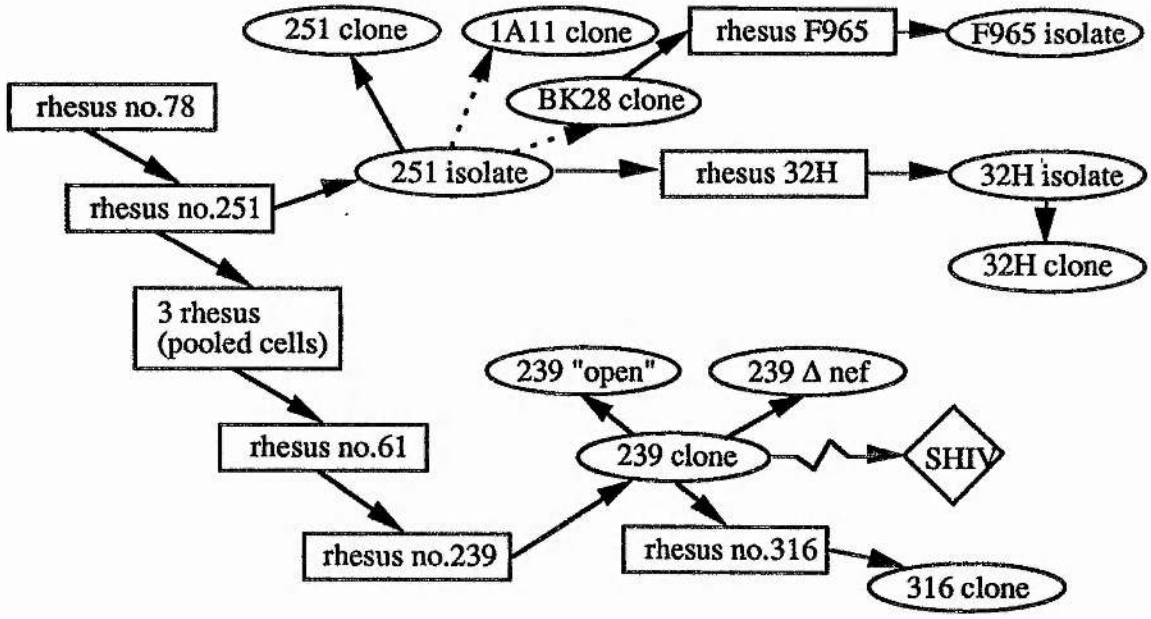


Figure 6. Derivation of SIVmac viruses.
Squares represent a passage of virus isolates through an animal and ovals individual virus isolates or clones. Where a dotted line is used, the exact derivation was impossible to trace. Modified from Schultz and Hu (1993).

isolated: SIVsm from sooty mangabeys (Fultz *et al.*, 1986), SIVmne from pig-tailed macaques (Benveniste *et al.*, 1988), SIVagm from African green monkeys (Fukasawa *et al.*, 1988) and SIVmnd from mandrills (Tsujimoto *et al.*, 1988). Fig. 6 shows the derivation of SIVmac isolates.

Simian and human immunodeficiency viruses and their infection of hosts have some remarkable similarities including virus morphology, genome organisation, (see also Fig. 7) life cycle, tropism, and pathology of disease they cause. The drawback of the SIV system is, that the SIV and HIV envelope glycoproteins are relatively distinct. Nevertheless, the model of SIV infection of macaques has been instrumental for testing a variety of vaccination strategies against lentiviruses, some of which are discussed below.

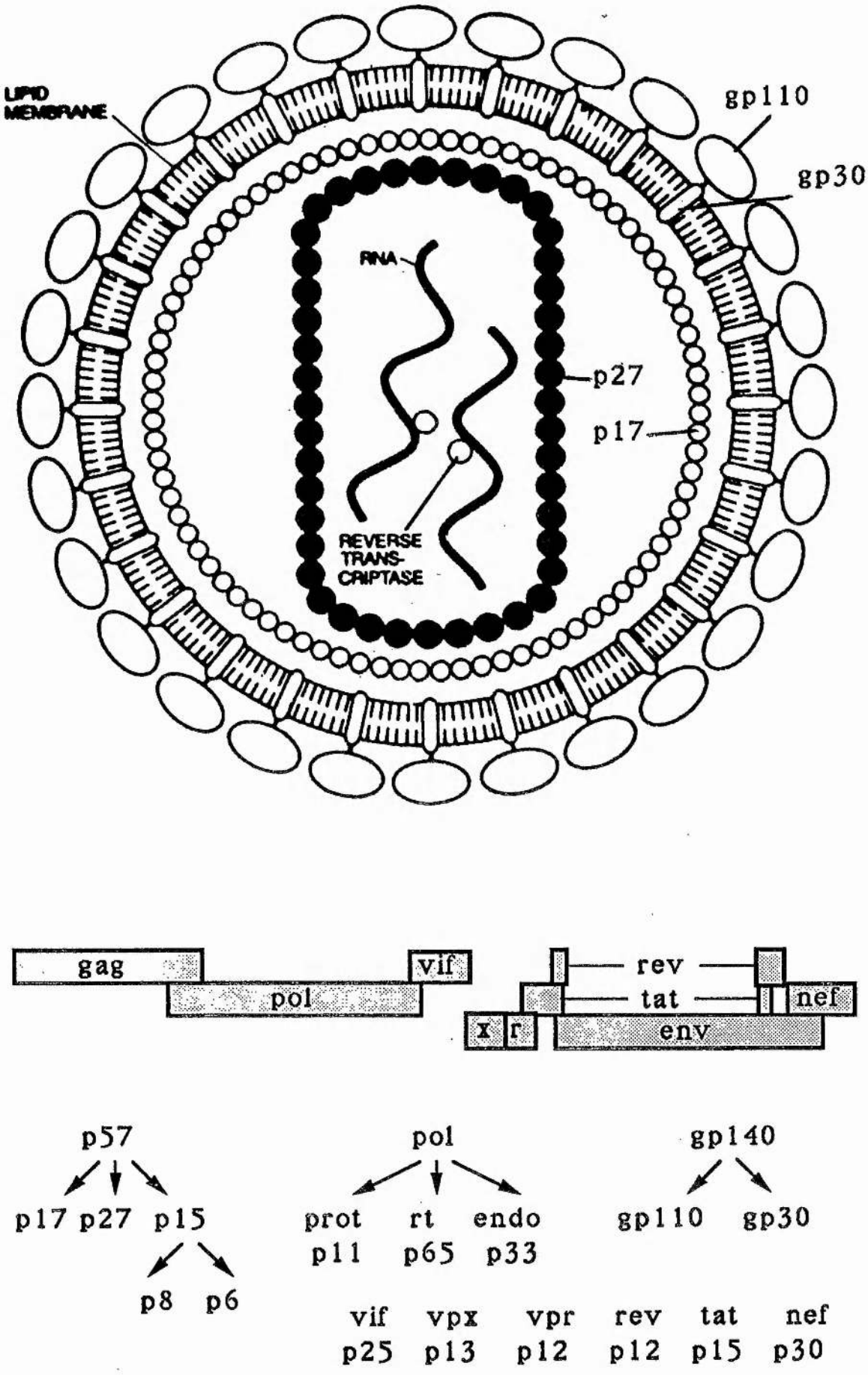


Figure 7. Particle and genome organization of simian immunodeficiency virus.

C.6.2.1 Whole inactivated virus vaccines.

Initially, the traditional vaccination approach was taken using the whole inactivated SIV vaccines. These early vaccinations delayed or even prevented the onset of disease and successfully protected a proportion of animals against infection (Desrosiers *et al.*, 1989; Sutjipto *et al.*, 1990; Murphy-Corb *et al.*, 1989.) The animals were challenged with 10 to 50 units of 50% of monkey infectious doses (MID_{50}) of a homologous SIV and the protected ones remained virus free for over a year (Stott *et al.*, 1990; Putkonen *et al.*, 1991; Johnson *et al.*, 1992a and b). Protection was also achieved against heterologous strains of SIV, but it did not extend to HIV-2 (Cranage *et al.*, 1992; Gardner and Murphy-Corb 1990; Johnson *et al.*, 1992a). However, when vaccination experiments were carried out using virus-infected cells, approximately half of the control animals vaccinated with uninfected cells were protected as well (Stott *et al.*, 1991). It was also found, that the vaccinated animals were protected against challenge with SIV grown in human, but not in simian cells (Langlois *et al.*, 1992; Cranage *et al.*, 1992, 1993). Thus, although there was an increased protection after vaccination with virus-infected compared to uninfected cells, the protection observed with whole inactivated virus vaccine and infected cells was almost certainly due to immune responses against cell components of the vaccines rather than against the virus antigens themselves (Stott *et al.*, 1993; Cranage *et al.*, 1992, 1993).

So which host cell proteins are responsible for the protection and what is their role in inactivated virus vaccines? The incorporation of MHC molecules into retrovirus particles has been known for some time (Bubbers and Lilly, 1977) and was confirmed for HIV and SIV (Gelderblom *et al.*, 1987). More recently, the heavy chain of HLA class I and both the α and β chains of HLA class II were identified in HIV-1 and SIV vaccine preparations (Cranage *et al.*, 1993). It was even estimated, that there is a 2-3:1 ratio of HLA DR:gp120 on the surface of purified virions (Arthur *et al.*, 1992). Antibodies specific for HLA class I, but not class II, molecules were found in animals vaccinated with inactivated virus preparations (Chan *et al.*, 1992; Cranage *et al.*, 1993). However, immunizations with mouse cells expressing either HLA class I or class II molecules protected against a human-grown SIV infection only one half of

the mouse cell/HLA class II-vaccinated macaques, while the other animals became infected (Stott, 1993). Passive transfer of sera from inactivated virus-immunized animals did protect naive recipients (Stott, 1993) and antibodies to HLA molecules, including β_2-microglobulin, do inhibit infection by HIV and SIV (Arthur *et al.*, 1992). It remains to be explained, why antibodies against host cell components present on the virus particles protected, while neutralizing antibodies against gp120 did not. On the other hand, sera from uninfected human cell-immunized and protected animals failed to neutralize the challenge SIV stock (Stott *et al.*, 1991). Thus, despite the puzzling data so far, the significance and potential of these observations for the development of human HIV vaccines should not be overlooked.

C.6.2.2 Live attenuated vaccines.

Live attenuated SIV vaccines were used in several instances. Immunization with live attenuated molecular clone 1a11 derived from SIVmac did not protected rhesus monkeys against infection with 100 to 1000 MID_{50} of pathogenic SIVmac251, but delayed disease and death (Marthas *et al.*, 1990). Similar results were reported after infection of cynomolgus macaques with avirulent strain of HIV-2 and a subsequent challenge with 10 to 100 MID_{50} of pathogenic SIVsm (Putkonen *et al.*, 1990). Nine months after the SIV challenge, the virus could be recovered from the lymph nodes of monkeys, but no disease developed. By large the most impressive protection against lentivirus infection has been achieved by vaccination with live attenuated SIVmac239, which carried a deletion in the *nef* gene (Daniel *et al.*, 1992). The animals were initially challenged with 10 MID_{50} of either virulent SIVmac239 or uncloned SIVmac251. The virulent viruses were never recovered from the vaccinated animals, which all remained healthy for 9 months. Two of the protected animals were re-challenged with 100 to 1000 MID_{50} of uncloned SIVmac251 with identical results. This efficient protection represents a unique opportunity to study and determine the correlates of protection, and identify the critical viral components for its induction.

C.6.2.3 Subunit vaccines.

SIV vaccine formulations based on recombinant proteins have been largely unsuccessful. In summary, SIVmac-derived gag, gag p55, p27, gp160 or gp120

produced in bacteria, yeast, insect cells, CHO cells or expressed by recombinant vaccinia virus have been used as immunogens, some induced strong T helper responses or neutralizing antibodies, but all have uniformly failed to induce protection against infection (reviewed in Stott, 1993). In this respect, the SIVmne virus seems to be exceptional, possibly due to the fact that SIVmne induces somewhat slower disease progression than the other SIV strains (Benveniste *et al.*, 1988). Thus, a combined vaccination scheme using a live vaccinia virus expressing gp160 followed by a boost with purified gp160 produced in the baculovirus system protected macaques against subsequent challenge with 1 to 9 MID_{50} of SIVmne (Hu *et al.*, 1992). However, this protection failed to be repeated in SIVmac (Mills *et al.*, 1992, 1993; Giavedoni *et al.*, 1993). In a second SIVmne study, immunization with four peptides corresponding to conserved SIV and HIV regions of gp160 fused to β-galactosidase did not prevent infection of SIVmne, but protected animals from disease progression for at least 3 years (Shafferman *et al.*, 1991). Moreover, the infection could not be transferred by inoculation of naive macaques with viable lymph node cells nor peripheral blood lymphocytes isolated from immunized and challenged animals (Shafferman *et al.*, 1992). In an extension of this work, inactivated plasma from the peptide-vaccinated or SIV-infected animals protected naive recipients from SIVmne infection and the same study suggested, that of the four peptides present in the vaccine mixture, the two originating from gp41 were responsible for inducing neutralizing (and anti-syncytial) activity (Lewis *et al.*, 1993). In all the above SIVmne experiments, the vaccine and challenge viruses were genetically matched.

Recently, chimeric SIV viruses carrying HIV-1 tat, rev, vpu and env genes were constructed and shown to replicate, although poorly, in cynomolgus macaques (Shibata *et al.*, 1991; Li *et al.*, 1992; Sakuragi *et al.*, 1992). Such chimeras used as challenge viruses, and even more so as immunogens, offer a system for testing or comparing efficacy of HIV-1-based vaccines in macaques and determining the correlates of protection.

D. OBJECTIVES OF THE WORK.

Successful vaccines against HIV may have to invoke both mucosal and systemic immunities probably by inducing both humoral and cell-mediated immune responses. The mechanisms underlying antigen processing and presentation to T cells together with the antigenic variability of HIV argue for incorporating more than one immunogenically relevant HIV determinant, i.e. more than one T cell site and/or antibody epitope, into such a vaccine. Thus, there is a need for vaccine design, that allows certain flexibility in terms of which and how much of HIV proteins the vaccine delivers. Also, the vaccine may have to incorporate engineered immunogens, which would avoid the undesirable determinants causing e.g. induction of Th2 at the expense of Th1 T cell subsets, immunosuppression, apoptosis or tissue damage. Moreover, should the vaccine contain non-replicating antigens, these will have to be introduced into the MHC class I presentation pathway for induction of MHC class I-restricted T cell responses. Finally, but not lastly, the vaccine should be relatively stable, cheap and easy to prepare. The use of solid matrix-antibody-antigen (SMAA) complexes as vaccine may have the potential to meet all the above requirements. Using the SIV model, the availability of separately prepared SIV antigens incorporated into SMAA complexes may allow both the evaluation of the efficacy of such complexes in preventing infection and/or disease in experimental animals and studying the roles of individual SIV proteins in induction of beneficial immune responses. Moreover, purified SIV proteins provide tools for analysis of humoral and T-helper response specificities induced during natural SIV infection and by any current or future protective vaccination schemes.

The work presented in this thesis has three principal aims: i) to develop SMAA complexes as a universal vaccine vehicle for delivery of antigens, ii) to clone all SIV genes, express the respective proteins and develop protocols for protein purification, and iii) to develop this vaccine design for potential use in humans, which involves humanizing the antibody used for the construction of SMAA complexes.

There will be no attempt to scale up the production of SIV antigens nor of the reshaped antibody.

In the first part, the feasibility of construction of SMAA complexes using a single tag-specific monoclonal antibody and multiple tag-linked antigens was demonstrated. It was shown that a 14-amino acid oligopeptide, present in the phospho (P) and V proteins of simian virus 5 (SV5) and designated Pk, retained its antigenicity when attached to the C-terminus of two 'foreign' proteins [p27 of SIV and glutathione-*S*-transferase (GST)] such, that these proteins could be incorporated into SMAA complexes using mAb SV5-P-k that had been originally raised against the native SV5 P and V proteins.

Secondly, all SIV unglycosylated proteins were cloned and expressed in bacteria. The expressed SIV proteins were flanked at their N-termini with either GST or a small histidine tag, and at their C-termini with the Pk tag. The two-tag protocol for purification significantly improved the purity of isolated proteins and assured their full-size. The second purification step involving the Pk tag and a tag-specific mAb incorporated the recombinant SIV proteins into complexes used as immunogens. The SIV *env* gene was inserted into a baculovirus vector such that the expressed virus glycoprotein was coupled at its C-terminus to the Pk tag.

The aim of this part of the work is to evaluate the importance of individual SIV antigens incorporated into SMAA complexes for inducting of protection of macaques against SIV challenge. Supposing vaccination with the mixture of all SIV proteins in SMAA complexes is protective, this simple and versatile system could be used to determine the minimum formula for a protective vaccine.

Finally, genes for the variable domains of heavy and light chains of SV5-P-k mAb were cloned. The variable regions were expressed in bacteria as Fab and Fv antibody fragments and their Pk tag specificities were confirmed in an ELISA assay. The murine complementarity-determining regions of the SV5-P-k mAb were then grafted onto suitable human frameworks and the whole reshaped mAb, now of the human IgG1 isotype, is currently being expressed in CHO cells.

MATERIALS AND METHODS

1. Plasmids.

Plasmid pBK28-SIV (Kornfeld *et al.*, 1987) containing the SIV (macaque) proviral DNA, derivative of the Mm251 isolate, inserted in pUC18 was kindly provided through the MRC AIDS Reagent Project. Plasmids pBluescript containing rev, tat and gp160 of SIVmac251(32H)(pJ5) were generous gifts of E. Rud (Wellcome Research Labs, Beckenham, Kent). Plasmids pGEX-2T and pQE-9 were purchased from Pharmacia (Milton Keynes, U.K.) and QIAGEN (Hybaid, Middlesex, U.K.), respectively. Vectors for the expression of Fab and scFv antibody fragments in bacteria were obtained from G.Winter (MRC Laboratory of Molecular Biology, Cambridge) and the eukaryotic vectors expressing immunoglobulin genes were gifts from B. Harris (Scotgen, Aberdeen).

2. Recombinant DNA.

Plasmid DNAs were prepared using the alkaline lysis method of Birnboim and Doly (1979) and treated by standard protocols (Maniatis *et al.*, 1982), of which some selected methods are described below. Enzymes used for recombinant DNA work were purchased from New England Biolabs. Inc. (NEB; Bishop's Stortford, Herts), Promega (Southampton), Bethesda Research Laboratories (Paisley) or Boehringer Mannheim (Lewes, East Sussex) and used according to the vendors' recommendations.

3. Agarose-gel electrophoresis.

DNA samples were analyzed by horizontal agarose-gel electophoresis. The concentration of agarose (Sigma) reconstituted in TBE buffer (90 mM Tris-borate, 2 mM EDTA pH 8.0) ranged from 0.8 to 4% (w/v) depending on the size of DNA analyzed. The size of the gel was 10 x 10 x 0.5 cm. DNA samples were mixed 1:5 (v/v) with 6x loading buffer [0.25% (w/v) bromophenol blue, 30% (v/v) glycerol] and loaded into the wells. The DNA species were gel-separated at 100 V for required times, stained with 1 $\mu g.ml^{-1}$ ethidium bromide in TBE buffer and visualized on a UV transilluminator.

4. Recovery of DNA fragments from agarose gels.

Stained agarose gels were placed onto a UV transilluminator and approximately 1-mm wide well was excised just in front of the desired DNA band. Gels were returned into the gel rig and TBE buffer was poured into the electrode tanks and gel wells, so that it did not overflow the surface of the gel. DNA band of interest was then electroeluted by 7 to 9 sequential 100-V 30-second pulses, each followed by removing and refilling the buffer from the excised well. The progress of electroelution was monitored on UV illuminator. The DNA-containing buffer aliquots were pooled, phenol/chloroform extracted, ethanol precipitated and dried. 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 recircularization during subsequent ligation reactions. Dried DNA was resuspended in 20 µl of water and a 2-µl sample was used to estimate the level of DNA recovery by gel electrophoresis.

5. Primers and DNA linkers.

Oligonucleotides used in this work were synthesized using an Applied Biosystems oligonucleotide synthesizer and phosphoramidite chemistry. Primers annealing at the 5'- and 3'-ends of SIV genes are designated -BACK (backward) and -FOR (forward), respectively, and their sequences are as follows: **nucBACK** 5'-GTG CAG GGA TCC ATG TTC TTG GAA AAG ATA GAG CCA GCA CAA G-3', **nucFOR** 5'-C TAG CTG AAT TCC TGC CAC CTC TCT AGC CTC TCC GG-3', **nefBACK** 5'-GTG CAG TGA TCA ATG GGT GGA GCT ATT TCC ATG AGG-3', **nefFOR** 5'-C TAG CTG AAT TCC GCC TTC TTC TAA CCT CTT CCT C-3', **15BACK** 5'-GTG CAG GGA TCC ATG GCA GAA GCC CTG AAA GAG GCC-3', **15FOR** 5'-C TAG CTG AAT TCC CTG GTC TCC TCC AAA GAG AGA ATT GAG-3', **17BACK** 5'-GTG CAG GGA TCC ATG GGC GCG AGA AAC TCC GTC TTG-3', **17FOR** 5'-C TAG CTG AAT TCC GTA ATT TCC TCC TCT GCC GCT AGA TGG-3', **27BACK** 5'-GCT CAG TGA TCA ATG CCA GTA CAA CAA ATA GGT-3', **27FOR** 5'-GAG ACA GGA TCC TAA TCT TGC CTT CTG TCC TGG-3', **polBACK** 5'-GTG CAG GGA TCC ATG GTG TTG GAA TTG TGG GAA GGA GGG ACA C-3', **protBACK** 5'-CTG CAG GGA TCC ATG CCT CAA TTC TCT CTT TGG AGG AGA CCA G-3', **protFOR** 5'-C TAG CTG AAT TCC AAG ATT TAG AGA CAT CCC CAG AGC-3', **revBACK** 5'-GTG CAG GGA TCC ATG AGC AGT CAC GAA AGA GAA GAA G-3', **revFOR** 5'-C TAG CTG AAT TCC GTC CTG AGG ACT TCT CGA CTT CTC-3', **rtBACK** 5'-GTG CAG GGA TCC ATG CCC ATA GCT AAG GTA GAG CCT GTA AAA GTC-3', **rtFOR** 5'-C TAG CTG AAT TCC GAG AAC TTG TCT AAT CCC CTG AC-3', **tatBACK** 5'-GTG CAG GGA TCC ATG GAG ACA CCC TTG AGG GAG-3', **tatFOR** 5'-C TAG CTG AAT TCC TCT GAA AAG GCC AGG AGC TGT TGC C-3', **vifBACK** 5'-CGT GAT GGA TCC ATG GAG GAG GAA AAG AAG TGG-3', **vifFOR** 5'-C TAG CTG AAT TCC TGC CAG TAT TCC CAA GAC CTT TGC-3', **vprBACK** 5'-CGT GAT GGA TCC ATG GAA GAA AGA CTT CCA GAA AAT GAA GGC-3', **vprFOR** 5'-C TAG CTG AAT TCC AGA GGG CGG TAT AGT TGA

GAG-3', **vpxBACK** 5'-CGT GAT TTA TCC ATG TCA GAT CCC AGG GAG AGA ATC CC-3', **vpxFOR** 5'-C TAG CTG AAT TCC TGA TAGTCC TGG AGG GGG AGG-3' and **GEXFOR** 5'-C GCT CTG TCG ACG CGC GCA AGC TTC AGA TCG TCA GTC AGT CAC GAT TAA TT-3'. Mutagenic primers used for the inactivation of protease were **mutprotBACK** 5'-A GTC TTA TTG AAT ACA GGG GC-3' and **mutprotFOR** 5'-GC CCC TGT ATT CAA TAA TAC T-3'. Oligonucleotides of positive and negative senses used for generation of the Pk tag-coding DNA linkers were: **GEXnPkpos** 5'-GA TCT GGA AGG CCG ATC CCA AAC CTT TTG CTG GGA TTG GAC TCC ACC G-3', **GEXnPkneg** 5'-GA TCC GGT GGA GTC CAA TCC CAG CAA AGG GTT TGG GAT CGG CTT TCC A-3', **GEXcPkpos** 5'-A ATT CCA GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TGA-3', **GEXcPkneg** 5'-A ATT TCA GGT GGA GTC CAA TCC CAG CAA AGG GTT TGG GAT CGG CTT TCC TTG -3', **HisPkpos** 5'-GA ATT CTC GGA AAG CCG ATC CCA AAC CCT TTG CTG GGA TTG GAC TCC ACC TAG-3' and **HisPkneg** 5'-AG CTT CTA GGT GGA GTC CAA TCC CAG CAA AGG GTT TGG GAT CGG CTT TCC GAG ATT TCT GCA-3'. The PCR BACK primers annealing to the leader sequences of the immunoglobulin chains were described previously by Jones and Bendig (1991) and the FOR primers used to amplify the variable regions were **κFOR** 5'-GGA TCC CGG GTG GAT GGT GGG AAG ATG-3' for the mouse κ light chain and **MoIgG2aFOR** 5'-GGA TCC CGG GAG TGG ATA GAC CGA TGG-5' for the mouse IgG2a heavy chain.

The primers used for the reshaping of the heavy chain were: **H1** 5'-CTG TCT CAC CCA GTG CAT TCC AAA ACT GGT GAA GGT ATA G-3', **H2** 5'-GTT CTT GGG GTT GTC TAG CGA TAT TGT AAA TCT GCC CTT CAC TGT GTC TCC ATA GTA GAT GGT AGT ACT GTC AGT ATT ATT GTA TGC AAC CCA CTC-3', **H3** 5'-CCC TTG GCC CCA GTA GTC AAA GCC GTA GTA AGG GCC CGC GCT TGC ACA AAA ATA G-3' and **H2.2** 5'-GTT GTC TCG CGA TAT TGT AAA TCT GCC CTT CAC TGT GTC TCC ATA GTA GAT GGT AGT ACT GTC AGT ATT AAT GTA TGC AAC CCA CTC AAG ACC-3'. The oligonucleotide H2, in addition to grafting CDR2, mutated Ser75 of the human framework to Pro.The

primers for reshaping of the light chain were: **L1** CTG CTG GTA CCA ATA CAA ATA AGT GAT GCC ATT ACT ATG TAC GAG ACT CTT ACT AGA CCT ACA GGT GAT GGT CAC-3', **L2** 5'-GCT TGG CAC ACC TGA GGC AAG GCT GGA CAT CTG GTA GAT CAG CAG-3' and **L3** 5'-CCC TTG GCC GAA CGT GAA TGG AAG TTC TAG AAT TTG ACC GCA GTA GTA GGT G-3'. The primers used for the construction of chimeric immunoglobulin genes were: **H-Pst** 5'- C ACC GCT CTC CTG CAG TTG GAC CTC-3', **L-PvuBACK** 5'-GAT ATT CAG CTG ACG CAG GCT G-3', **L-BamFOR** 5'-CGC GGA TCC AAC TGA GGA AGC AAA GTT TAA ATT CTA CTC CCG TCT TAT TTC CAA CTT TG-3'.

6. Isolation of polyA+ RNA from SV5-P-k hybridoma and generation of cDNA.

SV5-P-k hybridoma cells were harvested by centrifugation and washed with phosphate-buffered saline (PBS). The cell pellet (approximately 200 mg) was resuspended in 2.4 ml of GSS solution (5.5 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% (w/v) sodium lauryl sarcosine, pH 7.0), the DNA was sheared by passing through a 19-gauge needle several times and the lysate was spun in an eppendorf centrifuge. 2.5 ml of the resulting supernatant was loaded onto 2.7 ml of caesium trifluoroacetate solution [prepared by combining 25.5 ml of caesium trifluoroacetate in water, density 2.01 $g.ml^{-1}$ (CsTFA™ solution; Pharmacia cat. no. 17-0847-02) and 23.5 ml of 0.25 M EDTA pH 7.0] and separated by centrifugation at 31,000 rpm in Beckman SW 50.1 rotor at 15 °C for 24 hours. The total-RNA pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) and the polyA+ subpopulation was isolated on a oligo(dT) column using a Pharmacia 'mRNA Purification Kit' (cat. no. 27-9258-01). An oligo(dT) primer and avian myoblastoma virus (AMV) reverse transcriptase (Promega cat. no. M5101) were used for the first-strand DNA synthesis as recommended by the vendor.

7. Polymerase chain reaction.

A 50-µl PCR reaction mixture consisted of PCR buffer (final concentration: 50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100), 0.2 mM of each dNTP, 0.5 µM of each primer. The amount of template varied from approximately 0.1-10 ng of DNA. For the amplification of immunoglobulin variable regions, the PCR reaction mixture was briefly irradiated prior to addition of the cDNA template in order to inactivate any possible contaminating large-size DNA. The reaction mixture was overlayed with 2-3 drops of liquid paraffin and incubated for 3 min. at 94 °C before addition of the *Taq* DNA polymerase (Promega). Typically, 35 cycles of 94 °C for 1-2 min., 40-60 °C for 1-2.5 min. and 65-74 °C for 1.5-3 min. were carried out with the last cycle being 60 °C for 5 min.

8. Processing of PCR products for ligation.

After PCR amplification, the top liquid paraffin layer was removed and the reaction mixture volume increased to 100 µl with TE buffer. The PCR product was extracted with 100 µl of chloroform, precipitated by addition of 10 µl of 3 M sodium acetate and 250 µl of ethanol in -20 °C for 30 min., collected by centrifugation at 12,000*g* at 4 °C for 10 min., dried and resuspended in 20 µl of sterile water. The PCR-amplified DNA was digested with appropriate restriction endonuclease enzymes according to manufacturor's instructions for 1 to 16 hours. The digestion products were gel-purified (above) and used for ligation.

9. DNA ligation.

Approximately 400 ng of vector DNA and 40-400 ng of insert were combined in total of 20 µl of ligation buffer (50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 $mg.ml^{-1}$ bovine serum albumin) containing 200 U of

T4 DNA ligase (NEB) and incubated at 16 °C for 1 hour. The ligation mixture was used directly for transformation of competent *E. coli*.

10. Preparation of competent bacteria.

Escherichia coli strains DH1, JM101, M15, SG 13009, TG1 or RZ1032 were made competent using the modified RbCl method of Kushner (1978). Briefly, a 60-ml bacterial culture was grown to OD_{600} = 0.3-0.5, cooled on ice and spun at 756*g* for 10 min. The pellet was resuspended in 20 ml of ice-cold MOPS I solution (1 M MOPS pH 7.0, 100 mM RbCl), spun as above and resuspended in 20 ml of ice-cold MOPS II solution (1 M MOPS pH 6.5, 700 mM $CaCl_2$, 100 mM RbCl), spun as above and resuspended in 2 ml of MOPS II solution. Competent bacteria were used immediately for DNA transformation or stored at 4 °C for over a week.

11. Transformation of competent bacteria.

One half of the 20-µl ligation reaction or 10 µl of *in-vitro* mutagenesis reaction was combined with 100-200 µl of competent bacteria and incubated on ice for 30 min. with occasional end-over-end shaking. The bacteria were then heat-shocked at 42 °C for 2 min., returned to ice for 5 min. and plated. When selecting for transformants carrying antibiotic resistance-conferring plasmids, the transformation mix was diluted in 1 ml of Luria-broth (LB) medium and incubated at 37 °C for 1 hour, to allow for the expression of resistance-conferring gene, before plating on selection plates.

12. Preparation of single-stranded DNA.

Bacteriophage was added into 2-ml bacterial inoculum and the becteriophage-infected bacteria were grown for 6 to 16 hours at 37 °C. The bacteria were pelleted and 1.2 ml of the culture supernatant was transferred into a new eppendorf tube

containing 200 µl of 20% (w/v) polyethylene glycol 8,000 (PEG), 2.5 M NaCl solution, the tubes were vortexed and incubated at 20 °C for 15 min. The bacteriophage was pelleted in an eppendorf centrifuge, the supernatant was discarded and residual traces of PEG supernatant were removed after a re-centrifugation. The pellet was resuspended in 100 µl of TE buffer, thoroughly phenol/chloroform extracted, ethanol precipitated and dried. The ssDNA pellet was resuspended in 35 µl of water and its concentration was estimated by agarose-gel electrophoresis, as described earlier.

13. Oligonucleotide-directed mutagenesis of heavy and light chain variable regions.

Oligonucleotide-directed mutagenesis was described in detail by Kunkel and co-workers (1987). Briefly, bacteriophages M13mp18 containing human variable regions were grown in *dut⁻ ung⁻ E. coli*, strain RZ1032, in order to prepare uridine-containing ssDNA. (The incorporation of uridine into templates destabilizes the DNA when propagated in *dut⁺ ung⁺ E. coli* strains.) The extent of uridine incorporation into templates was estimated by the difference in plaquing efficiencies on *dut⁻ ung⁻* RZ1032 and *dut⁺ ung⁺* TG1 strains and only phages giving 10^5-10^6 less plaques on a TG1 bacterial lawn were used for preparation of templates. Three end-phosphorylated oligonucleotides corresponding to the murine complementarity-determining region DNA sequences were mixed with uridine-containing ssDNA template at ratios 10:10:10:1, allowed to anneal in a water-bath cooling from 70 °C to room temperature over 3 hours and transferred to ice for at least 10 min. To the annealing mixture, the following reagents were added: T7 DNA polymerase buffer (final concentration of 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol), 1 mM ATP, 0.2 mM dNTPs, 1 U of T7 DNA polymerase and 1 U of T4 DNA ligase in final volume of 100 µl. The extension reaction was carried out first on ice for 5 min., at room temperature for 5 min., at 37 °C for 30 min. and at 42 °C for 15 min. One fifth of the reaction volume was then used to transform competent TG1 bacteria

as described previously and plated. M13-containing plaques were picked and the bacteriophage DNA sequenced. The frequency of triple mutants, i.e. all 3 CDRs grafted in one reaction, was, on average, 10^{-1}.

14. DNA sequencing.

Single-stranded DNA templates were sequenced using the dideoxy chain-termination reaction. Sequenase® (Version 2.0) sequencing kit (United State Biochemicals) and [α^{35}S]dATP (10^3 Ci.mmol^{-1}; Amersham International) were used exactly according to the vendor's recommendations.

15. Production of stably transfected cell lines expressing heavy and light chains derived from the SV5-P-k antibody.

Approximately 1 million CHO cells was resupsended in 1 ml of serum-free G-MEM (GIBCO-BRL) medium. 15 µg of plasmid DNA was added to the cell suspension, the mixture was transferred to an electroporation cell and exposed to 250 V at 1180 µF capacitance using a Cell-Porator™ (BRL). Immediately after electroporation, the cells were placed into 10 ml of G-MEM + 10% (v/v) foetal-calf serum (FCS) in a 90-mm petri dish. The appropriate selection drugs were added after 1 day, i.e. 400 U.ml^{-1} of hygromycin B (Sigma cat. no. H-8272) for the light chain plasmids and 25 mg.ml^{-1} of mycophenolic acid (Sigma cat. no. M-3536) supplemented with 250 mg.ml^{-1} of xanthine (Sigma cat. no X-2001) for the selection of the heavy chain plasmids. In 10-14 days, individual colonies of resistant cells were ring-cloned, expanded and assayed for the expression of proteins of interest. Initially, stable cell lines expressing the light chains were isolated. The expression light chains were confirmed by a dot blot analysis of both the cell lysates (below) and tissue culture supernatants using horse radish peroxides (HRP)-conjugated anti-human kappa light chain antibodies (Sigma cat. no. A-7164) followed by ECL (Amersham International). Cell clones expressing the highest levels of light chains were then

transformed with the heavy chain genes, the expression of which was confirmed using HRP-conjugated protein A (Amersham International cat. no. NA9120) followed by ECL.

16. ELISA for detection of antibody H and L chain association, and cloned antibody or antibody fragment specificities.

ELISA plates were coated with approximately 2 μg of Pk tag-linked antigen in 100 μl phosphate-buffered saline (PBS) overnight at 4 °C. The wells were blocked with PBS + 10% (w/v) Marvel at room temperature for 1 hour, incubated with bacterial (for Fab and scFv fragments) or tissue culture (for recombinant antibodies) supernatants at room temperature for 1 hour and positive binding was detected with HRP-conjugated anti-human kappa light chain antibodies or HRP-conjugated protein A and solution of ImmunoPure® ABTS (2,2-azino bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) tablets (Pierce cat. no. 34026) and diluted hydrogen peroxide.

To confirm the association of light and heavy chains, the plate was coated with monoclonal anti-human IgG (Fc-specific) antibody (Sigma cat. no. I-6260), blocked as above and incubated with tissue culture supernatant followed by HRP-conjugated anti-human kappa light chain antibody and ABTS/hydrogen peroxide solution.

17. Isolation of recombinant baculovirus expressing SIV gp160.

Baculovirus expressing SIV gp160-Pk was generated by a co-transfection of *Bsu*36I-digested viral DNA and transfer vector pVL1393160Pk containing the SIV *env* gene into *Spodoptera frugiperda* cells IPLB-SF-21 (Sf 21) cells using lipofection. Briefly, approximately 10^6 Sf 21 cells from a spinner culture were placed into a 25-cm^2 flat in TC100 medium supplemented with 10% (v/v) FCS and allow to adhere for 90 min. The monolayer was washed 2x with 5 ml of OPTIMEM medium

(BRL-GIBCO) and 3 ml of the same medium was added to the cells. 0.5 μg of digested viral DNA was mixed with 2 μg of the transfer vector in 50 μl of water, the resulting mix was combined with an equal volume of Lipofectin (BRL cat. no. 8292SA), incubated at room temperature for 10 min. and the 100 μl Lipofectin/DNA solution was then added dropwise onto the cell monolayer. After 4 hours at 28 °C, the medium was replaced with TC100 + 10% (v/v) FCS and the cells were incubated at 28 °C for 4 days. Serial dilutions ranging 10^{0}-10^{-5} of the 4-day supernatant were used to infect Sf 21 cell monolayers for plaque purification of the virus. As described by O'Reilly and co-authors (1992), infected cell monolayers were overlayed with soft agar, stained with Neutral Red at 5 days post-infection and plaques were observed approximately 7 days later. Twenty plaques were picked and the viruses assayed for expression of the Pk tag in a Western blot analysis using SV5-P-k mAb. Virus stocks of two expressing isolates were gradually scaled up.

18. Antibodies and cells.

Monoclonal antibody SV5-P-k recognizing an epitope on SV5 phospho (P) and V proteins was isolated (Randall *et al.*, 1987) and its specificity was determined (Southern *et al.*, 1991) in our laboratory. Monoclonal antibodies KK33 (anti-SIV p27), KK7 (anti-SIV gp41/gp160) and KK8 (anti-SIV gp120/gp160) were obtained from K Kent [Medical Research Council AIDS Reagent Project (MRC ADP)]. Pooled antisera to SIVmac251(32H) from cynomolgous macaques were obtained from E.J. Stott (Potters Bar, U.K.) through the MRC ADP (ADP416). Serum from SIVmac251(32H)-infected macaques rhesus and C8166 cell lysate infected with the same virus were generous gifts from M. Cranage (CAMR, Port Down, U.K.). Chinese hamster cells (CHO-K1) cells (Kao and Puck, 1968) were grown in G-MEM medium (GIBCO-BRL) supplemented with 10% (v/v) FCS and insect Sf 21 cells were grown in TC100 medium (GIBCO-BRL) supplemented with 10% (v/v) FCS.

19. Screening for pQ9SIVPk recombinants by detection of expressed Pk tag on agar plates.

The gene for the Pk tag was positioned in plasmid pQ9cPk in such a way, that the Pk tag gene was out of the N-terminal His tag reading frame. This enabled screening for transformants carrying successfully inserted SIV gene by detection of the Pk tag. Thus, after ligation and transformation, individual bacterial colonies were induced by IPTG and screened directly on agar plates for the expression of the Pk tag. Plates with 18-hour-old colonies were covered with one sheet of nitrocellulose filter and two 3MM papers soaked in LB medium containing 100 $\mu g.ml^{-1}$ of ampicillin, 50 $\mu g.ml^{-1}$ of kanamycin and 1 mM IPTG, and incubated at 37 °C for 2 hours. Bacterial colonies attached to the nitrocellulose filter were disrupted by a 30-min. incubation in disruption buffer [4x concentrated: 20% (v/v) glycerol, 200 mM Tris-HCl, pH 7, 20% (v/v) 2-mercaptoethanol, 8% (w/v) sodium dodecyl sulphate (SDS)], the nitrocellulose filter was blocked with PBS + 20% (w/v) Marvel and processed as described for Western blots. After removal of the nitrocellulose filter, the plates were incubated at 37 °C for 2-3 hours in order to regenerate the bacterial colonies.

20. Expression and two-step purification of GST-SIV-Pk proteins.

GST fusion proteins were purified as described by Smith and Johnson (1988) with minor modifications. Briefly, bacteria carrying plasmids were grown to $OD_{600} = 0.7$ and the expression of the fusion proteins was induced by 1 mM IPTG at 20 °C for 3-6 hours. Bacteria were pelleted, washed in ice-cold TN buffer (20 mM Tris-HCl pH 7.8, 300 mM NaCl) and treated with 10 $mg.ml^{-1}$ of lysozyme on ice for 15 min. Triton X-100 was then added to final concentration of 1% (v/v) and the lysates were sonicated. The bacterial lysates were spun at 25,000*g* for 30 min. The supernatants were incubated with glutathione-agarose beads (sulphur linked, Sigma) rotating end-over-end at 4 °C for 1 hour. The beads were then transferred to a column,

washed with PBS and incubated with human thrombin (Sigma) to cleave off SIV-Pk antigens. Thrombin-released proteins were washed from the column by 2 column volumes of PBS and incubated with SV5-P-k-saturated particles of *S. aureus* on a rocking table at 4 °C for 1 hour and washed 4x with PBS.

21. Expression and two-step purification of His-SIV-Pk proteins.

His-SIV-Pk proteins were induced with 50 μM IPTG at 26 °C for 1-3 hours. Two procedures were used for protein purifications depending on whether the protein was soluble or insoluble.

i) Soluble proteins. Bacteria were pelleted, the pellet was washed in TN buffer, resuspended in the same buffer supplemented with 10 $mg.ml^{-1}$ of lysozyme and incubated on ice for 15 min. Triton X-100 was then added to final concentration of 1% (v/v), the lysates were sonicated and spun at 25,000*g* for 30 min. The supernatant was incubated with Ni^{2+}-nitrilotriacetic acid (NTA) resin and an excess of mAb SV5-P-k at 4 °C for 1-2 hours. The resin was transferred to a column, washed with 10 column volumes of TN buffer +1% (v/v) Triton X-100 and eluted with 1 volume of the TN buffer + 1% Triton X-100 supplemented with 250 mM imidazole. Eluted material was incubated with protein A-sepharose beads at 4 °C for 1 hour, washed with TN buffer + 1% (v/v) Triton X-100, resuspended in disruption buffer containing 0.25% (w/v) bromophenol blue and analyzed on 17% (w/v) SDS-polyacrylamide gels.

ii) Insoluble proteins. For His-vif-Pk, which was insoluble under the above conditions, the bacterial pellet was resuspended in buffer A [6 M guanidine hydrochloride (GuHCl), 0.1 M NaH_2PO_4, 10 mM Tris, 0.1% (v/v) Triton X-100, pH adjusted to 8.0 with NaOH] and stirred at room temperature for 1 hour. The lysate was then sonicated, spun at 25,000*g* for 30 min., the supernatant was incubated with a Ni^{2+}-NTA resin at 4 °C for 1-2 hours and the resin was transferred into a column. The column was washed with 10 column volumes of buffer A, 10 volumes of buffer B (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris, 0.1% (v/v) Triton X-100, pH

adjusted to 8.0 with NaOH immediately before use) and the His-vif-Pk protein was eluted with buffer E (same as buffer B, but pH adjusted to 4.5 with HCl immediately before use). Fractions containing the desired protein were pooled and dialyzed against 0.65 M NaCl, 20 mM Tris-HCl pH 7.8 and 0.1% (v/v) Triton X-100 overnight in order to remove urea. The amount of nickel affinity-purified protein was estimated on a Coomassie blue-stained gel and a 3-fold molar excess (antigen to antibody) of the purified His-vif-Pk was incubated with protein A-sepharose beads saturated with mAb SV5-P-k at 4 °C for 1 hour. The beads were washed in TN buffer + 0.1% (v/v) Triton X-100, resuspended in disruption buffer containing 0.25% (w/v) bromophenol blue and analyzed on a 17% (w/v) SDS-polyacrylamide gel.

22. Expression and two-step purification of SIV gp160-Pk.

500 ml of Sf 21 suspension culture (approximately 10^6 cells per ml) was infected with approximately 6 x 10^8 plaque-forming units of recombinant virus AcRP6-160Pk for 2 days, the cells were harvested and lysed in NP 40/DOC buffer [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% (v/v) sodium deoxycholate (DOC), 1% (v/v) Nonidet P-40 (NP 40)], sonicated and spun at 25,000*g* for 30 min. The supernatant was mixed with lentile lectin-sepharose beads at 4 °C for 1 hour, the beads were washed with NP 40/DOC buffer and loaded into a column, washed again with NP 40/DOC buffer and the bound glycoproteins were eluted with a high-sugar solution (500 mM methyl α-*D*-glucopyranoside, 500 mM methyl α-*D*-mannopyranosid in NP 40/DOC buffer). The eluate was directly incubated with mAb SV5-P-k complexed to a solid matrix for the second-step purification.

23. Construction of SMAA complexes.

SMAA complexes were prepared as described before (Randall and Young, 1988). Briefly, a 10% (w/v) suspension of 'fixed' and killed Cowan A strain of *Staphylococcus aureus* (Kessler, 1975) was incubated with an equal volume of

SV5-P-k antibody-containing ascitic fluids at 4 °C for 1 hour. Unadsorbed antibodies were removed by 3 consecutive pelletings (2,500*g* for 2 min.) and resuspensions in PBS. Antibody-saturated *S. aureus* particles were mixed with partially purified SIV-Pk or GST-SIV-Pk antigens at 4 °C for 1 hour and washed as above. Alternatively, particles of 'fixed' and killed *S. aureus* were incubated with SV5-P-k-His-SIV-Pk complexes eluted from the nickel-chelating column at 4 °C for 1 hour and the resulting SMAA complexes were washed with PBS as above.

24. SDS-PAGE and Western blot analysis.

Protein samples were suspended in disruption buffer containing 0.25% (w/v) bromophenol blue, boiled for 2 min. and individual polypeptides were separated on SDS-polyacrylamide gels crosslinked with 15% (w/v) *N,N*-diallyltartardiamide (DATD) using thin (0.75 mm) mini-slab gels of the Bio-Rad electrophoresis system. Separated polypeptides were either stained with Coomassie brilliant blue R-250 or transferred onto a nitrocellulose filter using a semidry gel electroblotter (LKB). The filters were blocked with PBS + 20% (w/v) Marvel and incubated with monoclonal or polyclonal antibodies ni PBS + 1% (w/v) Marvel. Bound antibodies were detected using either [^{125}I]protein A (Amersham International) in PBS + 1% (w/v) Marvel followed by autoradiography, or HRP-conjugated protein A (Amersham International) in PBS + 1% (w/v) Marvel followed by enhanced chemiluminiscence (ECL; Amersham International).

25. Dot blot analysis.

For testing the specificity of immune sera, nitrocellulose sheets were incubated with immunoaffinity-purified antigen (10-20 µg per 7x5 cm sheet) at room temperature for 1 hour and sandwiched between 84-well Terasaki plates containing 10 µl of diluted sera per well at room temperature for 30 min. The filters were then incubated with [^{125}I]protein A in PBS + 1% (w/v) Marvel followed by

autoradiography. Alternatively, PBS-soaked nitrocellulose filters were sandwiched between Terasaki plates containing 10 μl of transfected cell clone lysates in order to detect the expression of the immunoglobulin light chains. After a 30-min. incubation at room temperature, filters were blocked in PBS + 10% (w/v) Marvel and incubated with HRP-conjugated human κ light chain-specific mAb followed by ECL.

26. Immunization of mice.

To test the immunogenicities of recombinant SIV antigens assembled into SMAA complexes, mice were injected intraperitoneally with 200 μl aliquots of 0.5% (w/v) suspension of SMAA complexes, which contained 2-5 μg of recombinant SIV antigens. The immunizations were repeated after 2 to 3 weeks. 10 days after the second injection, mice were bled and the sera assayed for the presence of anti-SIV antibodies in ELISA or dot blot using recombinant SIV proteins and/or against Western blotted SIV-infected cell lysates.

RESULTS

A. CONSTRUCTION OF SMAA COMPLEXES USING A TAG-SPECIFIC MAB AND TAG-LINKED SIV P27.

Monoclonal antibody SV5-P-k was isolated in our laboratory, (Randall *et al.*, 1987) the binding of which to the original antigen, the phospho (P) protein of simian virus 5 (SV5), could be successfully competed with a nonapeptide (Southern *et al.*, 1991). The work presented in Part A of the Results demonstrates, that a short oligo-peptide tag, the core sequence of which corresponds to the originally competing nona-peptide, retained its antigenicity when attached to the C-terminus of SIV p27 and that the tag-linked p27 could be assembled into SMAA complexes using SV5-P-k mAb.

A.1. Construction of expression vector pGEX27Pk.

An *E. coli* expression vector pGEX-2T (Fig. 8) described by Smith and Johnson (1988) was chosen for the expression of Pk tag-linked p27 of simian immunodeficiency virus (SIV). In the pGEX-2T system, recombinant proteins are expressed as a fusion protein linked to the C-terminus of glutathione *S*-transferase and can be easily purified by affinity chromatography on immobilized glutathione. The GST gene is under control of the *lac* promoter and can therefore be induced by IPTG. The pGEX-2T vector was also designed so that the GST carrier polypeptide can be cleaved from the fusion protein by thrombin.

A region of the SIV *gag* gene encoding the major core protein p27 was amplified from plasmid pBK28-SIV DNA (Kornfeld *et al.*, 1987) using the

```
    ser gly lys pro ile pro asn pro leu leu

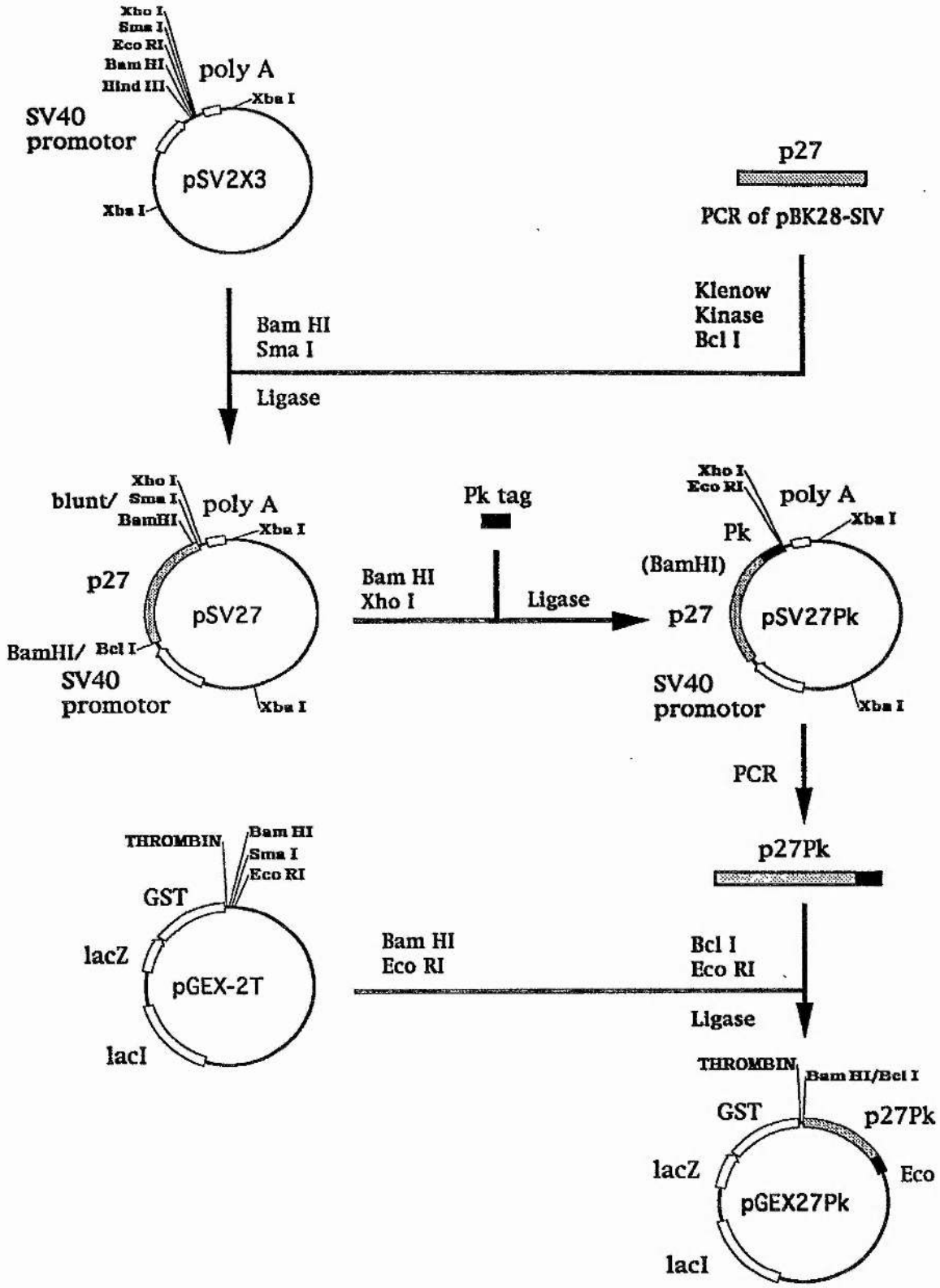
GA TCG GGA AAG CCG ATC CCA AAC CCT TTG CTG
     C CCT TTC GGC TAG GGT TTG GGA AAC GAC

    gly leu asp ser thr AMB OPA

    GGA TTG GAC TCC ACC TAG TGA ATT C
    CCC AAC CTG AGG TGG ATC ACT TAA GAG CT
```

Figure 9. DNA linker coding for the Pk tag.
The schematics shows sequences of positive and negative strand oligonucleotides, which were designed to give after annealing *Bam*HI-compatible and *Xho*I cohesive ends. The Pk tag linker codes for amino acids 95-108 derived from SV5 P and V proteins (bold). Underlined region corresponds to the synthetic nonapeptide, which competed with P and V proteins for binding to SV5-P-k antibody in a radioimmunoassay (Southern *et al.*, 1991).

Figure 8. Construction of pGEX27Pk expression vector.
(Next page) The gene coding for SIV p27 was amplified in a polymerase chain-reaction from pBK28-SIV plasmid. The PCR product was treated with polynucleotide kinase and the Klenow fragment of DNA polymerase, digested with *Bcl*I and the *Bcl*I/blunt fragment was ligated between the unique *Bam*HI and *Sma*I sites of the pSV2X3 polylinker (Hanke *et al.*, 1990). Resulting plasmid pSV27 was cleaved with *Bam*HI and *Xho*I for the ligation of the tag linker (Fig. 9). In order to regenerate *Bcl*I site at the 5'-end of the hybrid gene, p27-Pk DNA was amplified by PCR from pSV27Pk using 27BACK and the negative strand of the Pk tag linker as primers. Amplified p27-Pk DNA was digested with *Bcl*I and *Eco*RI restriction endonucleases and inserted into *Bam*HI and *Eco*RI sites of pGEX-2T yielding pGEX27Pk. Plasmid pGEXPk was constructed by an insertion of an *Eco*RI-digested Pk tag linker between the unique *Bam*HI and *Eco*RI sites of pGEX-2T.


Xho I
Sma I
Eco RI
Bam HI
Hind III
poly A
Xba I
SV40
promotor
pSV2X3
Xba I
p27
PCR of pBK28-SIV
Klenow
Kinase
Bcl I
Bam HI
Sma I
Ligase
blunt/
Xho I
Sma I
BamHI
poly A
Xba I
p27
pSV27
BamHI/
Bcl I
SV40
promotor
Xba I
Pk tag
Bam HI
Xho I
Ligase
Xho I
Eco RI
poly A
Xba I
Pk
(BamHI)
p27
pSV27Pk
SV40
promotor
Xba I
PCR
p27Pk
THROMBIN
Bam HI
Sma I
Eco RI
GST
lacZ
pGEX-2T
lacI
Bam HI
Eco RI
Bcl I
Eco RI
Ligase
THROMBIN
Bam HI/Bcl I
GST
p27Pk
lacZ
Eco
pGEX27Pk
lacI

polymerase chain reaction (PCR) and the p27 PCR product was ligated into vector pSV2X3 resulting in plasmid pSV27 (Fig. 8). An oligonucleotide linker encoding the tag antigen, i.e. 14-amino acids of SV5 P and V proteins (Fig. 9) recognized by monoclonal antibody SV5-P-k (Southern *et al.*, 1991), was inserted at the 3'-terminus of the p27 gene, yielding plasmid pSV27Pk. In order to insert the hybrid gene into pGEX-2T, the *BclI* site at the 5'-end of the p27-Pk gene had to be regenerated by PCR amplification from pSV27Pk using 27BACK primer and the negative strand of the Pk DNA linker as primers. The amplified sequences were inserted into *Bam*HI and *Eco*RI sites of pGEX-2T.

The Pk tag DNA linker was also inserted alone into pGEX-2T for the production of GST-Pk (not shown).

A.2. Expression of SIV p27-Pk and construction of SMAA complexes.

SIV p27-Pk was expressed in *E. coli* as a fusion protein with glutathione *S*-transferase. The expression of GST-p27-Pk was induced by addition of IPTG into bacterial cultures transformed with pGEX27Pk plasmid. Total bacterial lysates analysed by SDS-PAGE gave a prominent GST-p27-Pk band migrating at about 52 kDa (Fig. 10, lane L). For the purification of GST-p27-Pk, bacteria were lysed by incubation in TN buffer containing lysozyme followed by vigorous sonication. The sonicated lysate was centrifuged and the supernatant used for either glutathione-affinity column purification of GST-p27-Pk or construction of SMAA complexes.

Two approaches were taken to construct SMAA complexes. In the first, SMAA complexes were prepared by incubation of SV5-P-k-saturated *S. aureus* particles with the soluble antigen preparation. The resulting SMAA complexes containing GST-p27-Pk were washed several times in PBS, and analyzed by SDS-PAGE (Fig. 10, lane 1). The Coomassie blue-stained gel shows a highly purified GST-p27-Pk protein migrating between the heavy and light immunoglobulin chains of SV5-P-k antibody. All three bands are approximately equimolar, confirming that

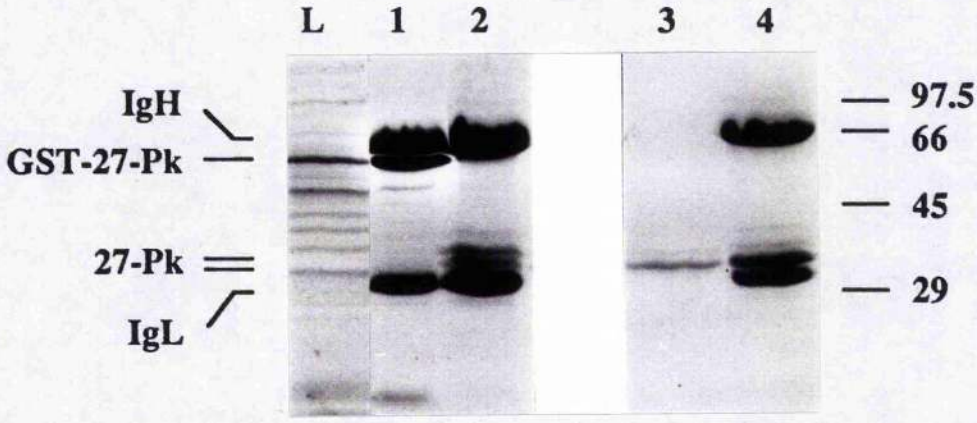


Figure 10. Purification of SIV p27-Pk (Coomassie blue-stained SDS-polyacrylamide gel).
Coomassie brilliant blue-stained SDS-polyacrylamide gel of a crude bacterial lysate after induction of GST-p27-Pk (lane L), SMAA complexes containing GST-p27-Pk (lane 1) and the same complexes, from which the GST domain was cleaved off with thrombin (lane 2). Also shown is a purified preparation of p27-Pk (lane 3) and SMAA complexes constructed using purified p27-Pk (lane 4). Polypeptides were separated by electrophoresis through a 12% (w/v) SDS-polyacrylamide minislab gel. The positions of immunoglobulin heavy (IgH) and light (IgL) chains, GST-p27-Pk, p27-Pk as well as the M_r markers of 97.4, 66, 45 and 29 kDa are shown. Note that purified p27-Pk runs as two bands, both of which react with mAb specific for p27 and for the Pk tag (Fig. 11).

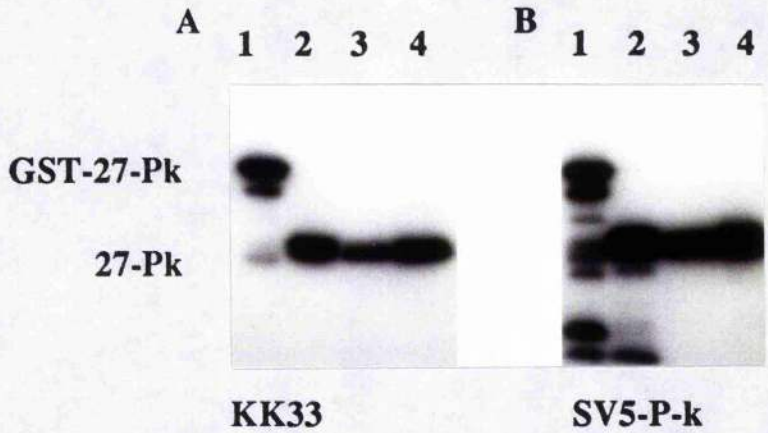


Figure 11. Western analysis of SIV p27-Pk-containing SMAA complexes.
The figure shows Western blot analysis of SMAA complexes probed with anti-p27 (KK33; panel A) or anti-Pk tag (SV5-P-k; panel B) mAbs followed by [^{125}I]protein A and autoradiography. SMAA complexes containing GST-p27-Pk are shown in lanes 1, the same complexes after thrombin removal of the GST domain are in lanes 2 and complexes containing a two step-purified p27-Pk (see Materials and Methods) in lanes 4. Lanes 3 contain purified p27-Pk eluted from the glutathione column.

SV5-P-k antibody in SMAA complexes was saturated with the antigen. In order to remove the GST domain of the fusion protein from the surface of the complexes, the GST-p27-Pk-containing SMAA complexes were incubated with thrombin and the cleaved complexes were analyzed by gel electrophoresis (Fig. 10, lane 2). The gel revealed a polypeptide doublet incorporated into the 'shaved' SMAA complexes. The reason for obtaining two bands after the thrombin digest of GST-p27-Pk is unclear, as amino acid sequence analysis of p27-Pk did not show any other obvious recognition sites for thrombin.

To confirm that the polypeptides incorporated in the SMAA complexes above were indeed SIV p27 linked to the oligopeptide Pk tag, disrupted SMAA complexes were analyzed by Western blotting. Incubation with both anti-Pk (SV5-P-k) and anti-p27 (KK33) mAbs resulted in a strong signal on the autoradiogram corresponding to the M_r of p27-Pk (Fig. 11, lanes 2). Shorter exposure of the same transfer showed that both bands of the doublet were recognized by both mAbs (not shown). The Western blot also revealed distinct faster-migrating bands detected with SV5-P-k (Fig. 11, right panel, lanes 1 and 2) and partially with KK33 (Fig. 11, left panel, lane 1) mAbs. These bands were products of proteolytic degradation, which had assembled into SMAA complexes via the oligopeptide tag. Taking advantage of the affinity of GST for its natural substrate glutathione, the contamination of SMAA complexes with degraded p27-Pk could be avoided by prior purification of GST-p27-Pk fusion protein on a glutathione affinity column. Crude bacterial lysate was incubated with glutathione coupled to agarose beads. Beads with adsorbed GST-p27-Pk were loaded into a column and thoroughly washed with PBS. As GST-p27-Pk bound to the beads via the GST end of the fusion protein, the washes removed all degradation products lacking the GST domain. p27-Pk was then released from the column by cleavage with thrombin (Fig. 10, lane 3). Note, that the thrombin cleavage of GST-p27-Pk adsorbed to the column again released a p27-Pk doublet. Purified p27-Pk readily assembled into SMAA complexes (Fig. 10, lane 4), this time without contamination from the degraded species (Fig. 11, lanes 4). Thus, a straightforward procedure has been

developed for producing relatively large amounts of tag-based SMAA complexes using SV5-P-k mAb and a bacterially expressed Pk-linked antigen.

A.3. Isolation of p27-specific monoclonal antibodies.

SMAA complexes containing recombinant SIV p27-Pk were used to immunize mice for the production of p27-specific monoclonal antibodies. Sera taken from immunized mice were tested for the presence of antibodies binding to p27-Pk and GST-Pk in a radioimmunoassay. The antibody titre against p27-Pk was much higher than that against GST-Pk (Fig. 12) demonstrating, that serum antibodies recognized the p27 portion of p27-Pk fusion protein rather than the Pk tag itself. Indeed, it was previously shown that immunization with SMAA complexes resulted in relatively fewer antibodies being produced to the epitope interacting with the antibodies in SMAA complexes compared with the other antigenic determinants on the incorporated protein (Randall and Young, 1988).

In experiments carried out by P. Szawlowski, splenocytes from immunized mice were isolated and used for the generation of hybridoma cells secreting SIV p27-specific antibodies. Seven independent cell lines producing antibodies to p27 were isolated in this manner. Fig. 13 shows the reactivates of these mAbs on a Western blot transfer of SIV-infected cell lysates. All mAbs recognized authentic p27 and were, apart from SIV-27-d, detected by [^{125}I]protein A. mAb SIV-27-d appeared negative, because it was of the IgG1 isotype and murine IgG1 isotype has a low affinity for protein A. Positive binding of SIV-27-d was demonstrated using polyclonal rabbit anti-mouse antibodies followed by [^{125}I]protein A (data not shown). Isotypes of the other p27-specific mAbs were: IgG2a (a, b, e and f), IgG2b (c) and IgG3 (g) (data not shown). The Western blot of p27-specific mAbs also showed weakly reacting bands migrating both slower and faster than p27, which probably correspond to other products of the gag polyprotein processing (Ferns *et al.*, 1987). The banding patterns suggest that there were three groups of mAbs (b and c grouped with KK33; e and f; and a and g), although this remains to be confirmed. None of the p27-specific mAbs

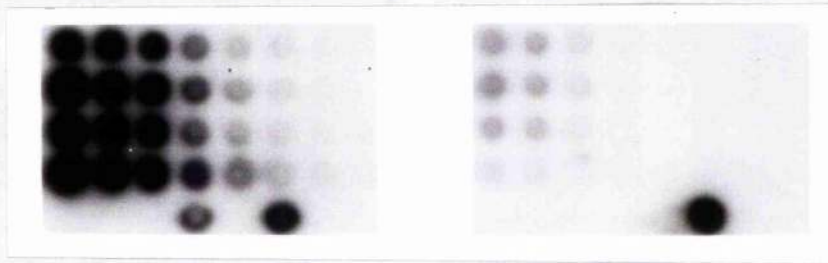


Figure 12. Immunogenicity of the Pk tag.
Nitrocellulose filters were coated with purified either p27-Pk or GST-Pk and incubated with immune sera from 4 individual mice followed by [^{125}I]protein A and autoradiography. As controls, single dots on the bottom row were reacted with p27-specific mAb (middle), SV5-P-k (right centre) and an irrelevant antibody (extreme right). The amounts of antigens bound to the nitrocellulose filters and times of exposure can be compared by the intensities of the SV5-P-k signals.

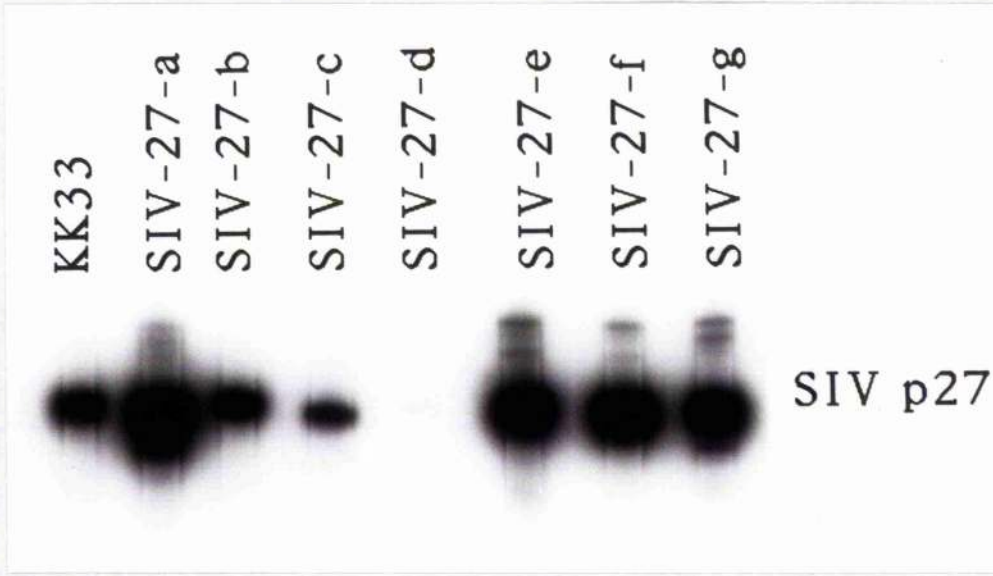


Figure 13. Reactivity of mAbs to authentic SIV p27.
A panel of seven mAbs induced by immunization with SMAA complexes containing recombinant SIV p27-Pk was assayed against SIVmac251-infected cell lysates. SIV-infected cell lysates were electrophoresed through a 15% (w/v) SDS-polyacrylamide minislab gel, transferred onto a nitrocellulose filter and incubated with corresponding ascitic fluids. The presence of bound mAbs was detected by [^{125}I]protein A followed by an autoradiography. p27-specific (KK33) mAb was used as a positive control, and SV5-P-k and irrelevant IgG2a mAbs served as negative controls.

reacted with SV5 proteins, i.e. did not recognize the tag peptide (data not shown). The isolation of p27-specific mAb demonstrates the general use of tag-based SMAA complexes for producing immunological reagents.

B. CLONING, EXPRESSION AND TWO-TAG PURIFICATION OF NON-GLYCOSYLATED SIV ANTIGENS.

In Part A, (i) a 14-amino acid Pk tag was identified, which retained its antigenicity when coupled to larger proteins, for example when attached to the C-terminus of simian immunodeficiency virus (SIV) p27. It was shown that (ii) a two-step purification protocol using N- and C-terminal affinity tags yields pure full-size p27-Pk. In part B, these observations were extended and further exploited for the incorporation of other non-glycosylated proteins of SIV into SMAA complexes.

B.1. Construction of universal pGEX-2T-derived vectors for addition of Pk tag

In order to facilitate the cloning and expression of other SIV proteins, two vectors, pGEXnPk and pGEXcPk, were constructed for attachment of the Pk tag at the N- and C-termini, respectively, to recombinant proteins. Both vectors were derived from the original pGEX-2T plasmid (see Fig. 34 and Table 2 for a schematic description of the resulting vectors). pGEXnPk was constructed by an insertion of a DNA linker coding for the Pk tag oligopeptide into the *Bam*HI site of the pGEX-2T polylinker in such a way, that the *Bam*HI site at the 5'-end of the tag linker was mutated while the 3'-end site was preserved for cloning. Thus the only restriction for the inserted genes is that they must have a 5' *Bam*HI site in a position which keeps the reading frame of GST-Pk. Similarly, pGEXcPk was constructed by inserting the tag linker into the *Eco*RI site of the pGEX-2T polylinker abolishing the 3'-end *Eco*RI recognition site, so that genes could be cloned between *Bam*HI and *Eco*RI. In the

pGEXcPk case, the amino acid codons of the Pk tag were immediately followed by a stop codon.

B.2. Cloning of SIV genes into pGEX-2T-derived expression vectors

The SIV genes encoded by a continuous open reading frame (ORF) were PCR amplified from plasmid pBK28-SIV (Kornfeld *et al.*, 1987). The BACK (5') PCR primers contained a recognition site for restriction endonuclease *Bam*HI and, if not already present in the gene sequence, an ATG codon was added at the beginning of the genes. The start codon was added for the possible future expression of the SIV genes in the absence an N-terminal tag. The *nef* gene contained an internal *Bam*HI site, so nefBACK primer carried a recognition sequence for *Bcl*I. The polBACK primer annealed to the beginning of the *pol* ORF. The FOR (3') primers created an *Eco*RI site. The gene for *tat* was derived from SIVmac(32H)(pJ5) proviral DNA (Rud, 1991; Rud *et al.*, 1992) and was PCR amplified from already cloned and spliced *tat* sequences in pBluescript. The resulting pGEX-2T-derived vectors carrying SIV genes are schematically described in Table 2.

B.3. Purification of recombinant Pk-linked SIV proteins expressed from pGEX-2T-derived vectors.

Expression of the recombinant proteins of the general structures GST-(thrombin site)-Pk -SIV or GST-(thrombin site)-SIV-Pk from the corresponding vectors were confirmed by a Western blot analysis using mAb SV5-P-k (Fig. 14). However, although successful purification of vpr-Pk and vpx-Pk was also achieved using this system (Fig. 15), the purification of most other SIV Pk-linked antigens proved problematic for a variety of reasons. In particular, difficulties were encountered in removing the GST domain with thrombin. For example, there was a thrombin cleavage site near the C-terminus of p17 (Randall *et al.*, 1993a). Other

Table 2. Construction of bacterial vectors expressing GST-SIV-Pk fusion proteins[a].

pGEXnPk[b]	GST-(*Bam*HI)-**Pk**-*Bam*HI,*Sma*I,*Eco*RI
pGEXcPk[c]	GST-*Bam*HI,*Sma*I,*Eco*RI-**Pk**-(*Eco*RI)
pGEXnefPk	GST-(*Bam*HI/*Bcl*I)-**nef**-*Eco*RI-**Pk**
pGEXnucPk	GST-*Bam*HI-**nuc**-*Eco*RI-**Pk**
pGEXNpolPk[d]	GST-*Bam*HI-**Npol**-*Eco*RI-**Pk**
pGEX15Pk	GST-*Bam*HI-**p15**-*Eco*RI-**Pk**
pGEX17Pk	GST-*Bam*HI-**p17**-*Eco*RI-**Pk**
pGEX27Pk[e]	GST-(*Bam*HI/*Bcl*I)-**p27-Pk**-*Eco*RI
pGEXrtPk	GST-*Bam*HI-**rt**-*Eco*RI-**Pk**
pGEXPktat	GST-(*Bam*HI)-**Pk**-*Bam*HI-**tat**-*Bam*HI,*Sma*I,*Eco*RI
pGEXvifPk	GST-*Bam*HI-**vif**-*Eco*RI-**Pk**
pGEXvprPk	GST-*Bam*HI-**vpr**-*Eco*RI-**Pk**
pGEXvpxPk	GST-*Bam*HI-**vpx**-*Eco*RI-**Pk**

a i) Where not already present, ATG was added at the beginning of the SIV gene. ii) All sequences coding for the C-terminal Pk tag are immediately followed by a stop codon. iii) DNA between the 3'-end of the GST domain and *Bam*HI site codes for a thrombin recognition site.

b Plasmid pGEXnPk was derived from pGEX-2T (Smith and Johnson, 1988) by inserting a DNA linker coding for the Pk tag into the *Bam*HI site. The Pk tag sequence is in the frame with GST.

c Plasmid pGEXcPk was derived from pGEX-2T (Smith and Johnson, 1988) by inserting a DNA linker coding for the Pk tag into the *Eco*RI site. The *Eco*RI site 3' of the Pk tag gene was mutated during the insertion, while the 5' *Eco*RI site was preserved for cloning. The Pk tag sequence is in the frame with GST.

d pGEXNpolPk contains DNA sequences from the very 5'-end of SIV pol ORF to the 3'-end of protease [nucleotides 2216 to 2827 of SIVmac251, clone BK28 as in the GeneBank database (Donahue *et al.*, 1988), i.e. the expressed protease domain contains Met plus 104 amino acids added to the N-terminus of the 100-amino acid viral protease.

e The construction of pGEX27Pk was described in Part A of Introduction.

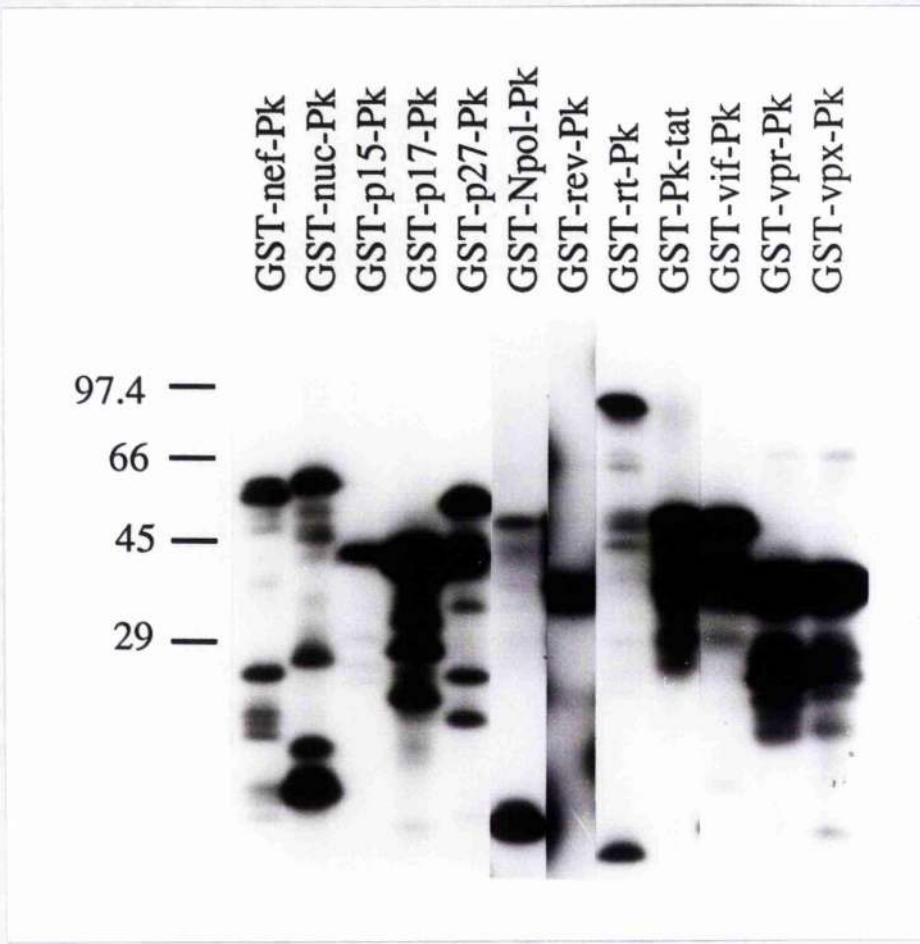


Figure 14. Western blot of bacterial lysates containing GST-SIV-Pk proteins. Bacterial cultures were grown to $OD_{600} = 0.7$ and induced with IPTG. Harvested bacteria were lysed, sonicated, boiled and loaded on a 15% (w/v) SDS-polyacrylamide gel. Separated polypeptides were transferred onto a nitrocellulose filter and the GST-SIV-Pk proteins detected using SV5-P-k mAb followed by [^{125}I]protein A and autoradiography. The M_r of the SIV proteins predicted from their amino acid sequences are: nef - 28.4 kDa, endonuclease (nuc) - 33.5 kDa, p15 - 16.0 kDa, p17 - 14.9 kDa, p27 - 25.5 kDa, N-terminal *pol*/protease - 21.9 kDa, rev - 13.4 kDa, reverse transcriptase (rt) - 64.6 kDa, tat - 14.7 kDa, vif - 25.3 kDa, vpr - 11.0 kDa and vpx - 12.9 kDa. M_r of the GST domain and the Pk tag are 26 kDa and 2.0 kDa, respectively. The positions of M_r markers in kDa are indicated.

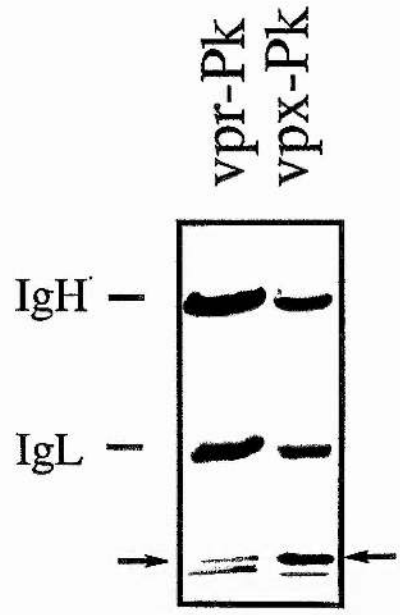


Figure 15. SMAA complexes containing vpr-Pk and vpx-Pk (Coomassie blue-stained SDS-polyacrylamide gel).
Recombinant GST-SIV-Pk proteins were first purified from crude bacterial lysates on a glutathione column via their N-terminal GST, digested with thrombin between the GST and SIV domains and the released SIV-Pk proteins were purified via the Pk tag during their assembly into SMAA complexes. The resulting SMAA complexes were analyzed on a 15% (w/v) SDS-polyacrylamide gel. On this percentage of gel, the vpr-Pk and vpx-Pk proteins migrated very similarly, but difference in their electrophoretic mobilities can be seen comparing the distances of their protein bands from the dye front. Also shown are the positions of the heavy (IgH) and light (IgL) chains of SV5-P-k mAb.

difficulties included low levels of expression (nef, nuc, p15 and rt) and protein insolubility (vif and, after removal of GST, endonuclease).

B.4. Cloning and expression of His-SIV-Pk proteins.

The main obstacles in the preparation of Pk-linked SIV antigens using pGEX vectors were low levels of expression in bacteria, proteolytic removal of the GST domain from the GST-SIV-Pk fusion proteins and insolubility. In an attempt to overcome some of these problems, the N-terminal GST domain was substituted by a histidine affinity tag (His). Histidine residues have a high affinity for metal cations, thus enabling the purification of proteins fused to a His tag by chelating immobilized metal ions (Smith *et al.*, 1988). Moreover, His-linked proteins may be purified under strong denaturing conditions, e.g. 8 M urea or 6 M guanidine hydrochloride, which helps to overcome protein insolubility problems. The affinity tag used in this work was a short 12-amino acid-long peptide containing 6 histidine residues.

The cloning of SIV genes into bacterial vectors expressing His-SIV-Pk fusion proteins is schematically represented in Table 3. Initially, a universal primer, GEXFOR, was designed containing a *Hin*dIII site, which annealed to the pGEXSIVPk plasmids immediately downstream of the Pk sequence. Using the GEXFOR primer and the respective BACK primers described above, genes for nef-Pk, nuc-Pk , p15-Pk, p17-Pk, rev-Pk, rt-Pk, vif-Pk and vpx-Pk were PCR amplified from the pGEXSIVPk vectors and inserted between *Bam*HI and *Hin*dIII sites of the pQE-9 (QIAGEN) plasmid polylinker (Table 3).

In order to simplify the addition of His and Pk tags at antigen termini, plasmid pQ9cPk was also constructed. The *Eco*RI site in the promoter region of pQE-9 was first mutated and a synthetic DNA linker coding for the Pk tag was inserted between the *Bam*HI and *Pst*I sites of the polylinker, generating plasmid pQ9cPk. In this vector, the Pk and His tag coding sequences are in different reading frames and are only brought into the same reading frame by insertion of the appropriate genes between the *Bam*HI and *Eco*RI sites. Thus the expression of the Pk tag can be used as a marker for

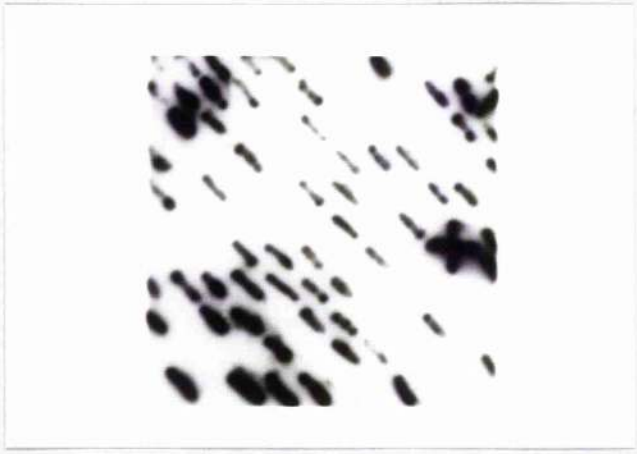


Figure 16. Screening of pQ9SIVPk clones by IPTG-induction of Pk tag-linked proteins on agar plate.
The Pk tag gene in pQ9cPk plasmid was out of the His tag reading frame, so the insertion of a SIV gene could be screened for by an expression of the Pk tag. Induced bacterial colonies were lifted onto a nitrocellulose filter, disrupted and probed with SV5-P-k mAb followed by [^{125}I]protein A and autoradiography.

screening bacterial colonies containing properly inserted His-SIV-Pk genes using mAb SV5-P-k (Fig. 16). Plasmid pQ9cPk was used for cloning of the *p27*, *rev*, *tat* and *vpr* genes. Because the expression level of GST-Npol-Pk was very low and one possibility was, that the protease cleaved itself out of the fusion protein and thus rendered the protein undetectable by SV5-P-k mAb, the protease enzymatic activity was inhibited by a substitution of Asp25->Asn in the active site of the enzyme (Swanstrom *et al.*, 1990). The mutated gene now designated as *Npol^{i}* was also cloned into the pQ9cPk vector (Table 3). The inactivation was achieved using mutagenic positive and negative sense PCR primers hybridizing across the active site region and a PCR assembly. All the above His-SIV-Pk proteins were induced by addition of IPTG to bacterial cultures and the crude lysates were analyzed on a Western blot using mAb SV5-P-k (Fig. 17). Non-degraded His-SIV-Pk proteins migrated at mobilities predicted from their amino acid sequences (see above), taking into account the addition of 1.4 kDa of His and 2 kDa of Pk tags. His-nef-Pk and His-nuc-Pk

Table 3. Construction of bacterial vectors expressing His-SIV-Pk fusion proteins[a].

pQE-9[b]	His-*Bam*HI,*Sal*I,*Pst*I,*Hin*dIII
pQE9nefPk	His-(*Bam*HI/*Bcl*I)-**nef**-*Eco*RI-**Pk**-*Hin*dIII
pQE9nucPk	His -*Bam*HI-**nuc**-*Eco*RI-**Pk**-*Hin*dIII
pQE915Pk	His -*Bam*HI-**p15**-*Eco*RI-**Pk**-*Hin*dIII
pQE917Pk[c]	His -*Bam*HI-**p17**-*Eco*RI-**Pk**-*Hin*dIII
pQE9rtPk[c]	His -*Bam*HI-**rt**-*Eco*RI-**Pk**-*Hin*dIII
pQE9vifPk	His -*Bam*HI-**vif**-*Eco*RI-**Pk**-*Hin*dIII
pQE9vpxPk[c]	His -*Bam*HI-**vpx**-*Eco*RI-**Pk**-*Hin*dIII
pQ9cPk[d]	His-*Bam*HI,*Sal*I,*Pst*I,*Eco*RI-**Pk**-*Hin*dIII
pQ927Pk	His-(*Bam*HI/*Bcl*I)-**p27-Pk**-*Eco*RI-**Pk**-*Hin*dIII
pQ9Npol^{i}Pk[e]	His -*Bam*HI-**Npol^{i}**-*Eco*RI-**Pk**-*Hin*dIII
pQ9prot^{i}Pk	His -*Bam*HI-**prot^{i}**-*Eco*RI-**Pk**-*Hin*dIII
pQ9revPk	His -*Bam*HI-**rev**-*Eco*RI-**Pk**-*Hin*dII
pQ9tatPk	His -*Bam*HI-**tat**-*Eco*RI-**Pk**-*Hin*dIII
pQ9vprPk	His -*Bam*HI-**vpr**-*Eco*RI-**Pk**-*Hin*dIII

a i) Where not already present, ATG was added at the beginning of the SIV gene. ii) All sequences coding for Pk tag are immediately followed by a stop codon.

b QIAGEN catalogue number 32049. SIV genes already linked to the Pk tag were inserted into pQE-9 vector using the *Bam*HI and *Hin*dIII sites of the polylinker.

c Genes coding for p17-Pk, rt-Pk, vpr-Pk and vpx-Pk were inserted into plasmid pQE-9 by J. Southern and R.E. Randall.

d Plasmid pQ9cPk was constructed by first mutating the *Eco*RI site in the promoter region of pQE-9 followed by an insertion of the Pk tag DNA linker between *Pst*I and *Hin*dIII sites. The Pk tag sequence is out of the His tag frame and is brought into the frame by cloning in the gene of a desired recombinant protein. SIV genes were cloned into pQ9cPk using the *Bam*HI and *Eco*RI sites of the polylinker.

e Npol^{i} is the same protein as Npol in Table 2, except that the protease activity was inhibited by Asp25->Asn (in Npol^{i} amino acid 130) substitution.

showed significant levels of degradation, while His-Npol^{i}-Pk gave a major band corresponding to the full-size protein suggesting that the inactivation of protease was indeed successful. Similarly to Fig. 14, the band intensities in Fig. 17 do not reflect the levels of expression of individual His-SIV-Pk proteins.

B.5. Optimization of His-SIV-Pk expression.

All the His-SIV-Pk proteins were initially induced by the addition of 1 mM IPTG at 26 °C for 3 hours. Their levels of expression varied remarkably, but were similar to those observed for their GST-SIV-Pk counterparts. While some proteins, such as His-p17-Pk and His-vpx-Pk, were abundantly expressed giving prominent bands on a Coomassie blue-stained gel of total cellular proteins, others, such as His-Npol^{i}-Pk, were barely detectable by Western blotting. It was, therefore, attempted to optimize the expression of individual proteins by adjusting the IPTG concentration, time of induction and temperature. Firstly, the effect of IPTG concentration and temperature on the levels of expression was tested by inducing the cultures for 1 hour at 37 °C and 26 °C with doses ranging from 10 μM to 1 mM. Although for a number of proteins no difference in the levels of induction was observed, in general the optimum induction was achieved by addition of 10-50 μM IPTG at 26 °C. Examples of dose-responses for His-Npol^{i}-Pk, His-p15-Pk and His-tat-Pk are shown in Fig. 18. The time of induction was also varied from 0.5 to 3 or 6 hours after addition of 50 μM IPTG at 26 °C. For His-nuc-Pk, His-rev-Pk, His-vpr-Pk and His-vpx-Pk, the amounts in total cell lysates remained unchanged over the tested period. The levels of His-nef-Pk and His-p15-Pk decreased with time, while the amounts of the remaining His-SIV-Pk proteins increased. Representative examples of His-Npol^{i}-Pk (decrease), His-vpr-Pk (constant) and His-tat-Pk (increase) are shown on Fig. 19. The data are summarized in Table 4. The supposedly higher producing *E. coli* strain SG 13009 (Gottesmann *et al.*, 1981) was also used to express the low-expression-level proteins, but in this host, the

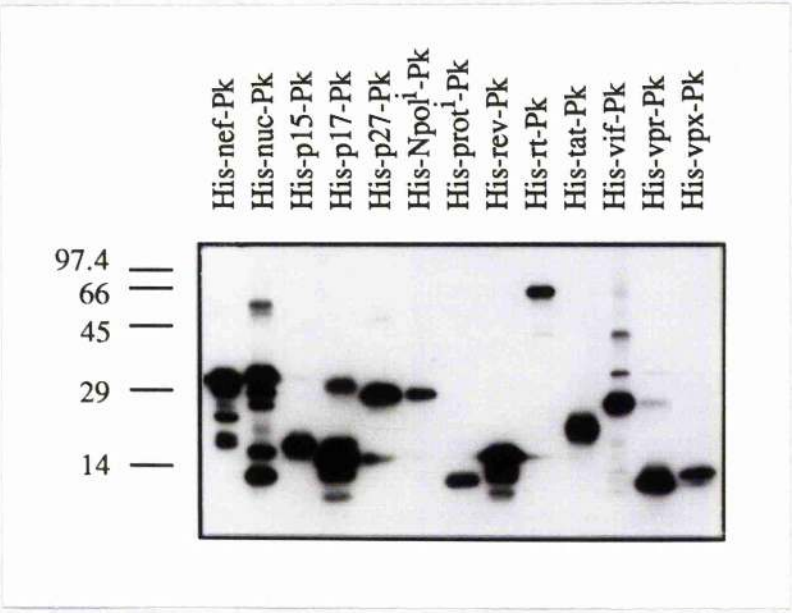


Figure 17. Western blot analysis of bacterial lysates containing His-SIV-Pk proteins.
Bacterial cultures were induced with IPTG, harvested, lysed, sonicated and boiled before loading onto a 17% SDS-polyacrylamide gel. Separated polypeptides were transferred onto a nitrocellulose sheet and probed with SV5-P-k mAb followed by [^{125}I]protein A and autoradiography. The positions of M_r markers in kDa are indicated.

expression was similar to that found with strain M15 (Villarejio and Zabin, 1974) (data not shown).

The levels of expressed His-Npol^{i}-Pk protein were consistently low, irrespective of which induction conditions or bacterial strains were used. It is possible that the translated, but non-functional N-terminal portion of the SIV *pol* polyprotein was inherently unstable. Therefore, using protBACK and protFOR primers, the gene of the inactivated protease (*prot^{i}*) was PCR amplified, cloned into the pQ9cPk vector (Table 3) and expressed as His-prot^{i}-Pk. However, the expression level of His-prot^{i}-Pk was not significantly increased (Table 4).

B.6. Purification of His-SIV-Pk proteins.

Bacterial cultures expressing individual His-SIV-Pk proteins were optimally induced with IPTG and the proteins were purified using a two-step purification

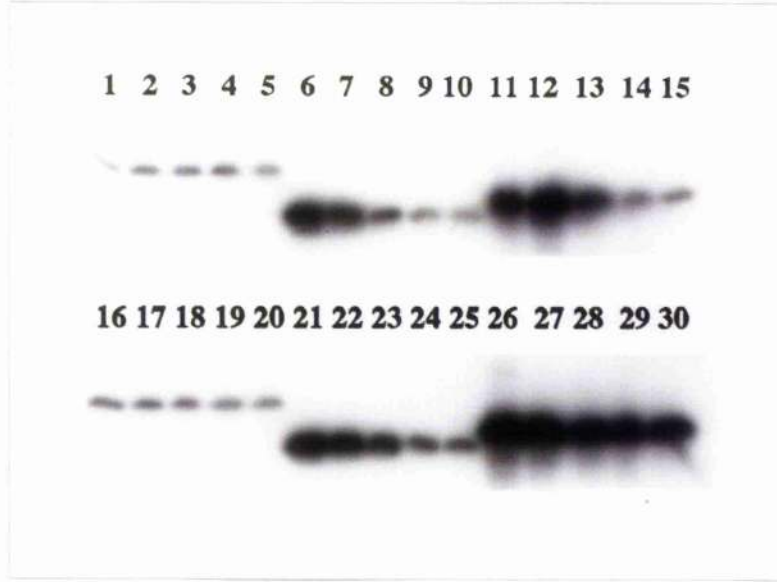


Figure 18. IPTG dose-response of His-SIV-Pk induction.
The figure shows 1-hour inductions of His-Npol^{i}-Pk (lanes 1-5 and 16-20), His-p15-Pk (lanes 6-10 and 21-25) and His-tat-Pk (lanes 11-15 and 26-30) at either 37 °C (lanes 1-15) or 26 °C (lanes 16-30) with the final concentration of IPTG of 10 μM (lanes 1, 6, 11, 16, 21 and 26), 50 mM (lanes 2, 7, 12, 17, 22 and 27), 100 μM (3, 8, 13, 18, 23 and 28), 500 μM (lanes 4, 9, 14, 19, 24 and 29) and 1 mM (lanes 5, 10, 15, 20, 25 and 30). His-SIV-Pk proteins were detected using SV5-P-k mAb followed by [^{125}I]protein A and autoradiography.

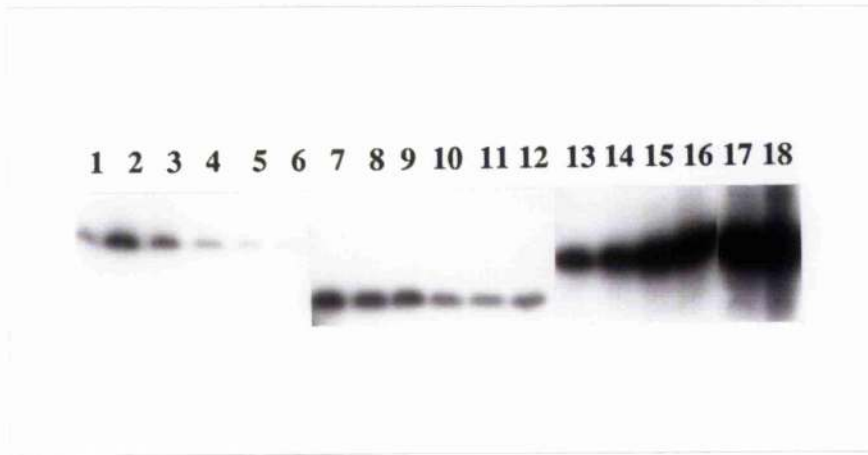


Figure 19. Time-course of His-SIV-Pk induction.
The figure shows inductions of His-Npol^{i}-Pk (lanes 1-6), His-vpr-Pk (lanes 7-12) and His-tat-Pk (lanes 13-18) with 1 mM IPTG at 28 °C for 1/2 hour (lanes 1, 7 and 13), 1 hour (lanes 2, 8 and 14), 2 hours (lanes 3, 9 and 15), 3 hours (lanes 4, 10 and 16), 4 hours (lanes 5, 11 and 17) and 6 hours (lanes 6,12 and 18). His-SIV-Pk proteins were detected using SV5-P-k mAb followed by [^{125}I]protein A and autoradiography.

Table 4. Expression levels of His-SIV-Pk fusion proteins.

	50 μM IPTG 26 °C 0.5-3 hours	optimum time (hour)	amount in total lysate at best
His-nef-Pk	dec	0.5	+++
His-nuc-Pk	const	0.5-3	+++
His-p15-Pk	dec	2	++
His-p17-Pk	inc	3	+++++
His-p27-Pk	inc	2-3	+++
His-Npol^{i}-Pk	dec	0.5-1	+
His-prot^{i}-Pk	const	3	+
His-rev-Pk	const	3	+++
His-rt-Pk	inc	3	+++
His-tat-Pk	inc	3	+++
His-vif-Pk	inc	3	++
His-vpr-Pk	const	2-3	+++
His-vpx-Pk	const	0.5-3	++++

a const - constant level; inc - increasing level over time; dec - decreasing level over time

b estimated levels of expression of fusion proteins in total bacterial lysates (protein mass per 1 litre of bacterial culture induced at $OD_{600} = 0.7$): + <10 $\mu g.l^{-1}$; ++ 10-200 $\mu g.l^{-1}$; +++ 200-700 $\mu g.l^{-1}$; ++++ 700 $\mu g.l^{-1}$ - 2 $mg.l^{-1}$; +++++ 10 $mg.l^{-1}$

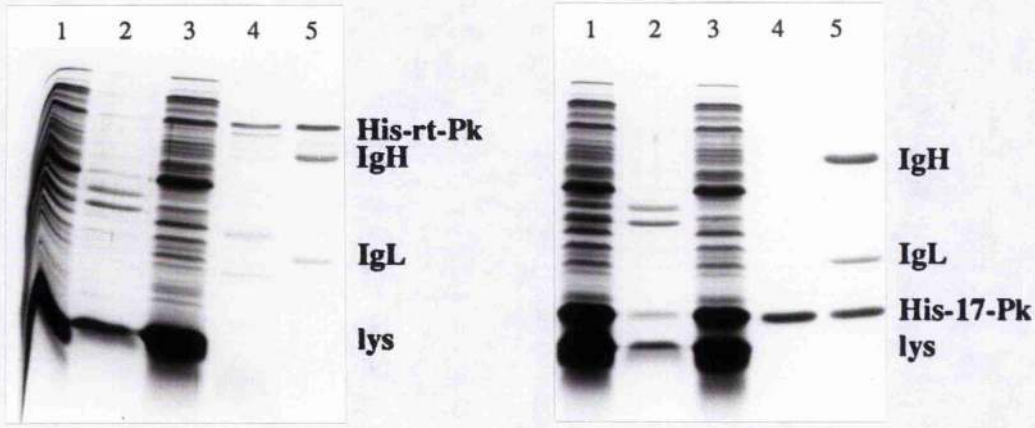


Figure 20. Two-step purification of His-rt-Pk and His-17-Pk (Coomassie blue-stained SDS-polyacrylamide gel).
The figure demonstrates a two-step affinity purification of His-rt-Pk (left) and His-17-Pk (right). Lanes 1: total bacterial lysates; lanes 2: insoluble fractions; lanes 3: soluble fractions; lanes 4: nickel column eluate; and lanes 5: SMAA complexes. The positions of both His-SIV-Pk proteins, the heavy (IgH) and light (IgL) chains of SV5-P-k mAb and lysozyme used to digest the bacterial wall are indicated. (The experiment was performed by R.E. Randall).

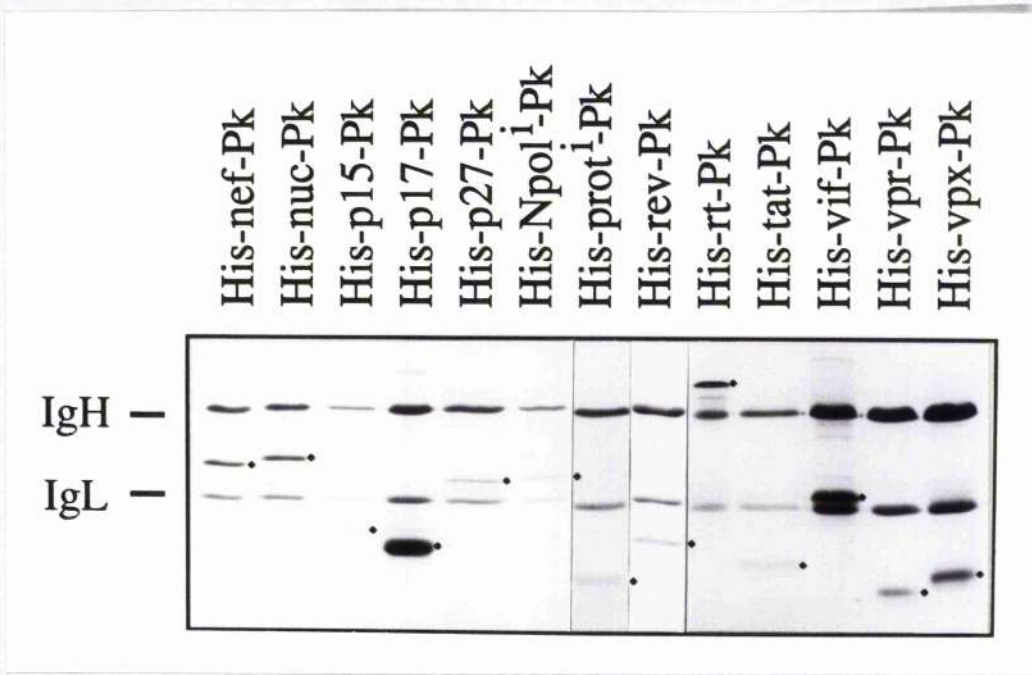


Figure 21. SMAA complexes containing His-SIV-Pk fusion proteins (Coomassie blue-stained SDS-polyacrylamide gel).
His-SIV-Pk proteins were purified using the two-step purification protocol and the resulting antigen-antibody complexes were visualized on a 17% (w/v) SDS-polyacrylamide gel stained with Coomassie brilliant blue. The heavy (IgH) and light (IgL) chains of SV5-P-k mAb are designated and the positions of His-SIV-Pk are indicated by diamonds.

protocol. For soluble proteins, bacterial lysates were incubated with an excess Pk-specific mAb SV5-P-k and the Ni^{2+}-NTA resin was added to the mixture. The beads were then loaded into a column and the unbound proteins (including the unreacted mAb SV5-P-k) were removed by washing. Bound antibody-antigen complexes were eluted with 250 mM imidazole. The eluates were then incubated with protein A-sepharose beads and the bound material analyzed on a Coomassie blue-stained 17% (w/v) SDS-polyacrylamide gel. The two-step purification of proteins for immunization purposes is illustrated for His-rt-Pk and His-17-Pk in Fig. 20.

A modified procedure was used for the purification of the insoluble His-vif-Pk protein. The first purification step on the nickel affinity column was carried out under denaturing conditions. His-vif-Pk was transferred to the column in 6 M guanidinium hydrochloride, this was exchanged for 8 M urea, in which the protein was eluted by lowering the pH. Nickel-purified His-vif-Pk was dialyzed overnight to remove urea, incubated with SV5-P-k-saturated protein A-sepharose beads and the antibody-antigen complexes were analyzed by SDS-PAGE. The composite of SMAA complexes containing all of the His-SIV-Pk proteins is shown in Fig. 21.

B.7. Analysis of antibodies to non-glycosylated SIV proteins in sera of SIV-infected macaques.

Sera taken from SIV-infected monkeys were tested in a Western blot against approximately equimolar amounts of individual recombinant proteins (as judged by Western blot analysis using the mAb SV5-P-k). The result using pooled sera (ADP416) from cynomolgus macaques infected with live SIVmac251(32H) is shown in Fig. 22. Antibody responses could be readily detected to endonuclease, p15, p17, p27, rt, and vif. Weak responses were also detected against vpx and protease (Fig. 22, right panel), but no reactivity was noted against nef, vpr, rev or tat. In contrast to the results presented in Fig. 22, there appeared to be similar levels (relative to each other) of antibodies to p15, p17 and p27 (data not shown), in addition to antibodies specific

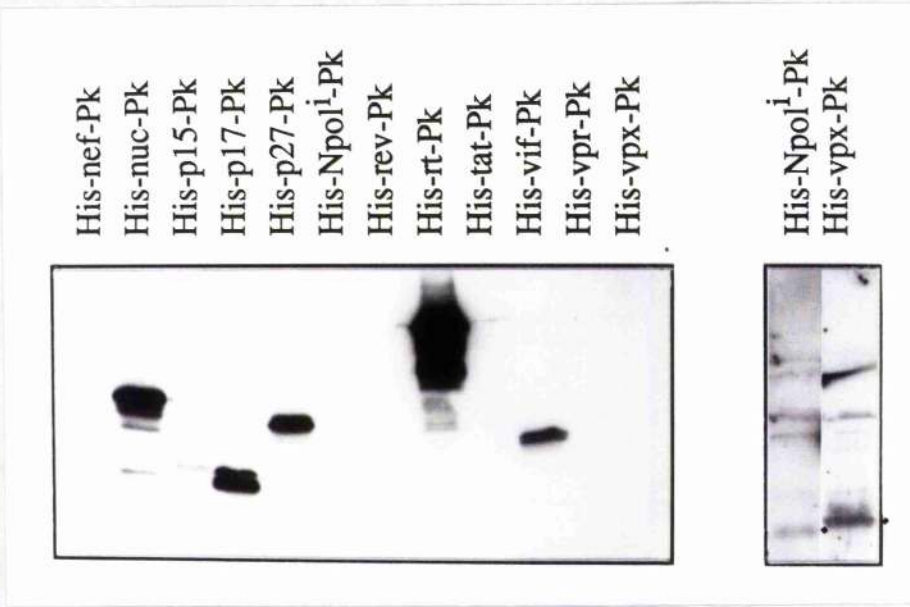


Figure 22. Analysis of sera from SIV-infected macaques.
The gel shows the reactivity of pooled sera taken from SIV-infected macaques with total *E. coli* cell lysates expressing individual recombinant SIV proteins. Antibodies were detected by HRP-conjugated protein A followed by ECL. Approximately equimolar amounts of the SIV proteins (as estimated by previous Western blot analysis using SV5-P-k mAb) were separated by electrophoresis through a 17% (w/v) SDS-polyacrylamide gel. The right hand panel shows a longer exposure of two lanes from the left panel, where specific bands corresponding to the appropriate His-SIV-Pk proteins are designated by diamonds.

to rt and vif, in a serum taken from macaque rhesus infected with SIVmac251(32H), but again no antibodies were detected to nef, vpr, rev or tat.

C. Cloning, Expression and Two-Step Affinity Purification of SIV Envelope Glycoprotein in Insect Cells.

Part C of the Results describes expression of recombinant SIV envelope glycoproteins linked at their C-termini to the Pk tag. After abortive attempt to express the *gp120-Pk* gene in bacteria, the *gp160-Pk* gene was inserted into a baculovirus vector and expressed in insect cells. This insect cell-produced glycoprotein was purified in a two-step purification protocol. The first step employed binding of gp160-Pk to a lectin column via its polysaccharide moieties followed by assembly of

Figure 23. Western blot analysis of total cellular lysates of bacteria expressing SIV *gp120Pk* gene.
Four cultures of independent bacterial clones carrying plasmid pGEX120Pk were induced by addition of IPTG, the bacteria were lysed, the lysates were Western blotted and probed with SV5-P-k mAb followed by [^{125}I]protein A and autoradiography. The position of GST-gp120-Pk is indicated.

the partially purified glycoprotein into SMAA complexes via the Pk tag and mAb SV5-P-k.

C.1. Expression of the SIV *env* gene in bacteria.

The sequence coding for the glycoprotein gp120 of SIV *env* was PCR amplified from pBK28-SIV plasmid, coupled to the Pk tag linker and inserted into pGEX-2T vector in a similar fashion as described in Fig. 8 for p27. However, when bacterial cultures transformed with the pGEX120Pk plasmid were induced with IPTG, the expression of GST-gp120-Pk did not result in a prominent band on Coomassie blue-stained SDS-polyacrylamide gels (not shown). Western blot analysis of four independent clones carrying pGEX120Pk using mAb SV5-P-k showed that the vast majority of GST-gp120-Pk was proteolytically degraded (Fig. 23). The absence of glycosylation in prokaryotic cells presumably had affected the gp120 folding and consequently protein stability and protease resistance.

C.2. Construction of a recombinant baculovirus expressing SIV gp160-Pk.

A recombinant baculovirus carrying hybrid *gp160Pk* gene in the position of the polyhedrin gene was constructed as follows. A DNA linker containing *Bam*HI and *Sac*I restriction endonuclease sites and a coding sequence for the Pk tag was first inserted between the *Bam*HI and *Pst*I sites in the polylinker of transfer vector pVL1393, generating plasmid pVL1393cPk. The *env* gene of SIVmac251(32H)(J5) was excised from a pBluescript plasmid-based construct (Rud, 1991) using *Bam*HI and *Sac*I endonucleases and cloned into the corresponding sites in the pVL1393cPk plasmid. The *Sac*I site lay just within the *env* gene so that the DNA coding for the last three C-terminal amino acids (ThrLeuLeu) of gp160 was deleted. The resulting plasmid pVL1393-160Pk was co-transfected with AcRP6-SC baculovirus DNA, linearized by *Bsu*36I endonuclease digest into Sf 21 cells as described by Kitts and colleagues (1990). Clearly separated plaques on the cell monolayer were selected and the virus clones were used for subsequent infections. To test for the expression of gp160-Pk, Sf 21 cell monolayers were infected with the individual virus clones. Two days post-infection, the cells were lysed and the cell lysates analyzed on a dot blot using mAb SV5-P-k to detect the Pk tag-linked env. All the virus clones tested expressed gp160-Pk and the infection of one, designated AcRP6-160Pk, was gradually scaled up to 500-ml suspension culture. The highest levels of gp160-Pk in both adhered and suspension cell culture were found at two days post-infection (Fig. 24). On this Western blot, mAb SV5-P-k readily detected two bands in total cellular lysates. One band was broader and migrated at around 160 kDa, which is in a good agreement with the expected glycosylated form of env. The faster-migrating band is of uncertain origin, but is probably a partially degraded species, as it seemed to migrate much slower than the unglycosylated env precursor, which, from its amino acid sequence, has a predicted M_r of 53 kDa.

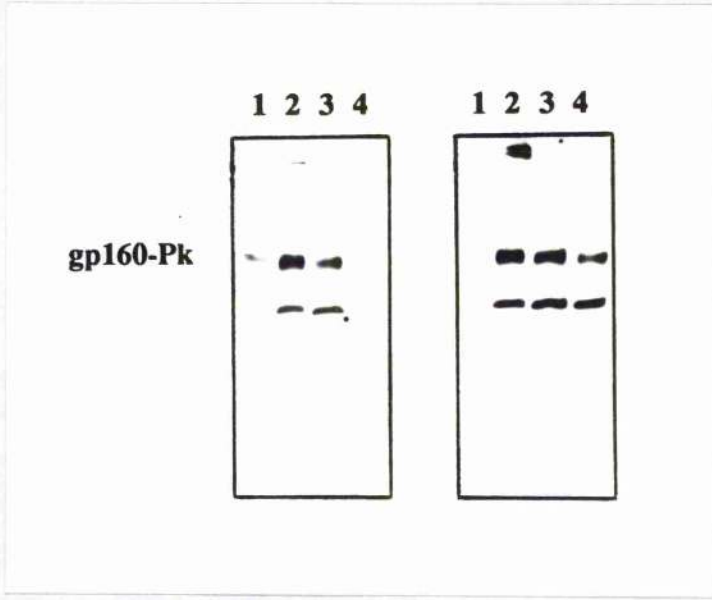


Figure 24. Time-course of SIV gp160-Pk expression in 500-ml culture of Sf 21 cells.
An Sf 21 cell monolayer (left) or a 500-ml suspension culture of Sf 21 cells at approximately 10^6 cells per ml (right) were infected at m.o.i. of 1-10 for 1 to 4 days (lanes 1 to 2). At each time point, cells were harvested, lysed and analysed on a Western blot using SV5-P-k mAb followed ECL.

C.3. Two-step purification of SIV gp160-Pk.

It is imperative for the induction of neutralizing antibodies, that the fidelity of surface structures presented by vaccines is preserved during the purification procedure. The affinity of lectins for polysaccharides and the elution of bound material from the lectin column by monosaccharides represent a suitable system for the first purification step, which avoids 'harsh' treatments of purified proteins, such as changes of pH or red-ox conditions, often needed for elution from other affinity matrices, that could alter the protein structure. The potential of different lectins for the first-stage purification of gp160-Pk was therefore examined.

A 500-ml suspension culture of insect cells was infected with virus AcRP6-160Pk at 1 to 10 multiplicity of infection and the cells were harvested after 2 days.

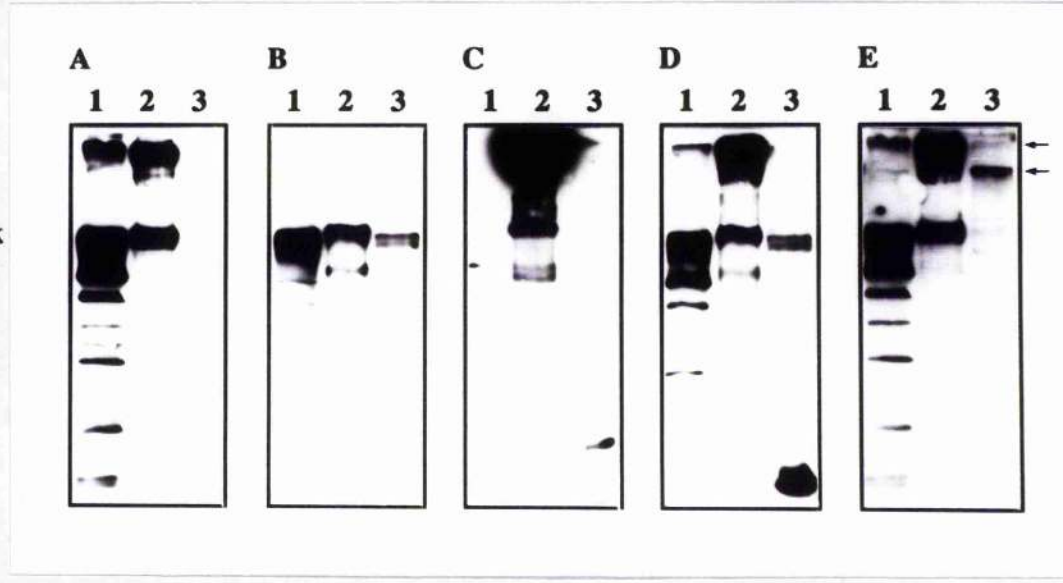


Figure 25. Western blot analysis of antigenicity and immunogenicity of baculovirus-expressed SIV gp160-Pk.
Polypeptides from total cell lysate of recombinant baculovirus AcRP6-160Pk-infected Sf 21 insect cells (lanes 1), insect cell-produced lentile lectin-purified SIV gp160-Pk (lanes 2) and total cell lysates of SIV-infected C8166 cells (lanes 3) were separated through a 10% (w/v) SDS-polyacrylamide gel, transferred onto a nitrocellulose filter and incubated with anti-Pk tag (panel A), anti-gp120/gp160 (panel B), anti-gp41/gp160 (panel C) mAbs, serum from SIV-infected macaque (panel D) or pooled sera from mouse immunized with SMAA complexes containing gp160-Pk (panel E) followed by HRP-conjugated protein A and ECL. Although the quantitative estimations of proteins are limited using ECL, panel C is a 5x-longer exposure relative to the other panels. The position of gp160 monomer is indicated.

The cells were lysed and the expressed SIV gp160-Pk in the soluble fraction was tested for carrying polysaccharide moieties by employing several types of lectin columns. These included lentile lectin (L), wheat germ agglutinin (WGA), succinylated wheat germ agglutinin (sWGA) and concanavalin A (ConA). Bound material was eluted using a combination of two monosaccharides, 500 mM methyl α-*D*-glucopyranoside and 500 mM methyl α-*D*-mannopyranoside. Although WGA appeared to bind the highest level of Pk tag-linked species, the greates yield, resulting from the most efficient release of gp160-Pk from the lectin column, was achieved using the lentile lectin (data not shown). Thus, the yield of purified gp160-Pk after the first purification step was approximately 400 μg per 500 ml of suspension culture.

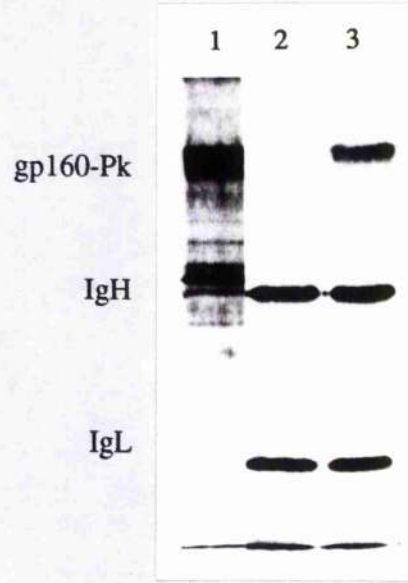


Figure 26. SIV gp160-Pk in SMAA complexes (Coomassie blue-stained SDS-polyacrylamide gel).
The first purification step of insect cell-produced SIV gp160-Pk on lentile lectin co-purified other glycosylated species (lane 1). These were removed during the second purification step, which incorporated gp160-Pk into SMAA complexes (lane 3). Monoclonal antibody SV5-P-k-saturated particles of *S. aureus* were analyzed on lane 2. The samples were separated through a 12% (w/v) SDS-polyacrylamide gel. The positions of gp160-Pk, and the heavy (IgH) and light (IgL) chains of SV5-P-k are indicated.

The gp160-Pk purification using a lentile lectin column was analyzed by a Western blot analysis (Fig. 25). The soluble fraction of the total baculovirus-infected insect cell lysates (lanes 1) was compared with the lentile lectin column eluate (lanes 2) and an SIV-infected cell lysate (lanes 3). These blots were probed with SV5-P-k, anti-gp120/160 and anti-gp41/gp160 mAbs in panels A, B and C, respectively, and with serum from an SIV-infected macaque (panel D). As is clearly demonstrated in panel A, the soluble fraction of total insect cell lysate was heavily contaminated with degraded gp160-Pk species, which were largely, but not entirely (panels B, C and D), removed during the lectin purification step.

In the second purification step, the gp160-Pk-containing eluate from the lentile lectin column was purified via its C-terminal Pk tag. Because the relatively high concentration of monosaccharides present in the elution buffer did not appear to

interfere with antibody binding, the eluate was directly incubated with 'fixed' and killed *S. aureus* particles saturated with SV5-P-k mAb. The second purification step resulted in assembly of SIV gp160-Pk into SMAA complexes (Fig. 26). The lectin eluate contained a number of species that co-purified with gp160-Pk from the affinity column (lane 1), however, the incorporation into SMAA complexes specifically selected for gp160-Pk (lane 3).

C.4. Fidelity of insect cell-produced SIV gp160-Pk.

To evaluate the immunogenic potential of the insect cell-produced SIV gp160, gp160-Pk was assembled into SMAA complexes and used for the immunization of mice. The immune sera were pooled and tested against both insect cell-produced gp160-Pk and SIV-infected cell lysates on a Western blot (Fig. 25, panel E). The mouse serum readily reacted with the immunogen, but there was only very weak recognition of the authentic envelope monomer in SIV-infected C8155 cells (panel E, lane 3). In addition to the monomeric form, there were also two slowly-migrating bands (indicated by arrows) in both insect and mammalian cell lysates, which might correspond to the glycoprotein dimers and tetramers. The notion, that these bands corresponded to ordered structures rather than protein aggregates, was further supported by the fact, that these species were detectable in insect cell-lysates using monkey immune serum (panel D), and Pk tag- (panel A) and gp41- (panel C), but not gp120-specific mAbs (panels B). Ordered complexes may completely hide an epitope, but a 'random' protein aggregate would probably leave at least some of the gp120 epitopes available for antibody recognition. If this was indeed the case and the bands represented dimers and tetramers, then incubation with mouse immune sera gave a much stronger signal with the tetramer compared to the dimer in insect cell lysates (panel E, lane 1), but the opposite was found for the mammalian-produced gp160 (panel E, lane 3), which gave a fainter tetramer signal. Differences in signal intensities most likely reflect the relative amounts of glycoprotein present in dimers and tetramers in the lysates, rather than reflecting differences in the induced antibody

specificity or the availability of a particular epitope on these oligomeric structures. The prevalence of the gp160-Pk tetramer over dimer in insect cell lysates is also suggested using SV5-P-k mAb and immune monkey serum (panels A and D, lanes 1 and 2).

An additional observation from this series of Western blots was, that gp41-Pk was not detected at all among lectin-purified glycoproteins and in insect cells (panel A, lane 2 and panel C, lanes 1 and 2). Gp41 was present in small amounts in mammalian cells, but was visualized only after a prolonged exposure (panel C, lane 3). Together with the failure to detect any gp120 (panel B, lane 3), this may suggest an inefficient proteolytic processing of SIV gp160-Pk/gp160 to gp120 and gp41-Pk/gp41 in insect cells and, at least, in the sample of SIV-infected C8166 cells used in this experiment. As for the insect cells, it was reported previously, that the inefficient cleavage of insect cell-produced HIV gp160 was due to a lack of the furine protease in this system (Morikawa *et al.*, 1993). In mammalian cells , the proteolytic cleavage of gp160 is a late processing event in the glycoprotein biogenesis and, as such, may account for the low proportion of cleaved gp160 in SIV-infected cells (Hallenberger *et al.*, 1992). However, there may be another indication, that the SIV-infected cell lysate was indeed a poor sample. Although a strongly-detected fast-migrating band was identified for the *gag* gene products, there was no apparent signal corresponding to rt (panel D, lane 3), an antigen normally detected in SIV-infected cell lysates by Western blotting. Also, it can be noted on the Western blot probed with the gp41/gp160-specific mAb, that this antibody recognized only very weakly the gp160- Pk monomer in insect cell lysates (panel C, lane 1), but did give slightly stronger signal, corresponding to the full-size protein, after lectin purification (panel C. lane 2). This may be a result of gp160-Pk concentration following lectin column chromatography. Of course, the limitation of a Western blot analysis is, that it detects only structures present on linear stretches of the protein backbone, while all discontinuous epitopes are lost. Nevertheless, SMAA complex-generated murine antibodies did recognize a band possibly corresponding to the dimer of authentic SIV

envelope. The weak signal against authentic monomeric SIV gp160 may reflect differences in insect vs. mammalian glycosylation patterns of proteins.

D. HUMANIZING OF PK TAG-SPECIFIC MAB SV5-P-K.

Part D of the Results describes humanizing of the murine Pk tag-specific monoclonal antibody SV5-P-k. In the first stage, the genes for the SV5-P-k heavy and light variable regions were cloned from the appropriate hybridoma cells and their sequences were determined. In the second stage, only the SV5-P-k complementarity-determining regions were grafted onto human immunoglobulins. The production of stably transfected CHO cells expressing the whole humanized Pk tag-specific mAb is currently under way.

D.1. Cloning of the SV5-P-k variable regions and expression of Pk tag-specific Fab and scFv fragments of antibody in *E. coli.*

In the first stage of the Pk tag-specific mAb reshaping, genes for the variable regions of the heavy and light chains were cloned and their DNA sequence determined. The immunoglobulin variable regions were PCR amplified from cDNA made from SV5-P-k murine hybridoma polyA$^+$ RNA. Primers used in the PCR reaction were a mixture of upstream primers specific for the leader sequences of heavy and light chains (61 and 405 primers, respectively; Jones and Bendig, 1991) and one downstream primer hybridizing to conserved nucleotide sequences of the constant regions of IgG2a and kappa. Amplified variable sequences were initially cloned into M13 phage for sequencing. Once the nucleotide sequences were determined (Fig. 27), specific PCR primers were designed, that only amplified the variable regions and generated the correct restriction endonuclease sites for cloning the genes into chimeric Fab (murine variable/human constant domains) and single-chain-Fv antibody fragment expression vectors (Fig. 28). Both Fab and scFv fragments were expressed in *E. coli* and their positive binding of the Pk tag, tested in

```
1/1                                     31/11
GTC GAC atg agg tgc ttt gct cag ctt ctg ggg CTG CTT GTG CTC TGG ATC CCT GGA TCC
val asp met arg cys phe ala gln leu leu gly leu leu val leu trp ile pro gly ser

     5'-gat att GAG CTC acg cag gct g-3'
61/21                                   91/31
ACT GCA GAT ATT GTG ATG ACG CAG GCT GCA TTC TCC AAT CCA GTC ACT CTT GGA ACA TCA
thr ala asp ile val met thr gln ala ala phe ser asn pro val thr leu gly thr ser

121/41                                  151/51
GTT TCC ATC TCC TGC AGG TCT AGT AAG AGT CTC GTA CAT AGT AAT GGC ATC ACT TAT TTG
val ser ile ser cys arg ser ser lys ser leu val his ser asn gly ile thr tyr leu

181/61                                  211/71
TAT TGG TAT CTG CAG AAG CCA GGC CAG TCT CCT CAC CTC CTG ATT TAT CAG ATG TCC AGC
tyr trp tyr leu gln lys pro gly gln ser pro his leu leu ile tyr gln met ser ser

241/81                                  271/91
CTT GCC TCA GGA GTC CCA GAC AGG TTC AGT GGC AGT GGG TCA GGA ACT GAT TTC ACA CTG
leu ala ser gly val pro asp arg phe ser gly ser gly ser gly thr asp phe thr leu

301/101                                 331/111
AGA ATC AGC AGA GTG GAG GCT GAG GAT GTG GGT GTT TAT TAC TGT GGT CAA ATT CTA GAA
arg ile ser arg val glu ala glu asp val gly val tyr tyr cys gly gln ile leu glu

                    3'-gc ccc tgt ttc GAG CTC tat tct-5'
361/121                                 391/131
CTT CCA TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AGA CGG GCT GAT GCT GCA CCA
leu pro phe thr phe gly ser gly thr lys leu glu ile arg arg ala asp ala ala pro

421/141
ACT GTA TCc atc ttc cca cca tcc aCC CGG G
thr val ser ile phe pro pro ser thr arg
```

Figure 27a. Sequences of the light chain PCR product amplified from SV5-P-k hybridoma cDNA.
The lower case in the DNA sequences indicates the primer-annealing regions, thus, although the primers specific for the leader sequences were degenerate, the sequence may not necessarily be genuine. PCR primers used for cloning of the variable regions into *E. coli* vectors expressing Fab and scFv fragments of antibody are shown above the sequences.

```
1/1                                          31/11
GTC GAC atg gac tcc agg ctc aat tta gtt ttc ctt GTC CTT ATT TTA AAA GGT GTC CAG
val asp met asp ser arg leu asn leu val phe leu val leu ile leu lys gly val gln

     5'-gtg cag CTG CAG gag tca ggg gga ggc tta gtg c-3'
61/21                                        91/31
TGT GAT GTG CAG GTG GTG GAG TCA GGG GGA GGC TTA GTG CAG CCT GGA GGG TCC CGG AAA
cys asp val gln val val glu ser gly gly gly leu val gln pro gly gly ser arg lys

121/41                                       151/51
CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGT TTT GGA ATG CAC TGG GTT CGT CAG
leu ser cys ala ala ser gly phe thr phe ser ser phe gly met his trp val arg gln

181/61                                       211/71
ACT CCA GAG AAG GGG CTG GAG TGG GTC GCA TAC ATT AAT ACT GAC AGT ACT ACC ATC TAC
thr pro glu lys gly leu glu trp val ala tyr ile asn thr asp ser thr thr ile tyr

241/81                                       271/91
TAT GGA GAC ACA GTG AAG GGC CGA TTC ACC ATT TCC AGA GAC AAT CCC AAG AAC ACC CTG
tyr gly asp thr val lys gly arg phe thr ile ser arg asp asn pro lys asn thr leu

301/101                                      331/111
TTC CTG CAA ATG ACC AGT CTA AGG TCT GAG GAC ACG GCC ATG TAT TAC TGT GCA AGT GCG
phe leu gln met thr ser leu arg ser glu asp thr ala met tyr tyr cys ala ser ala

          3'-g ccg aaa ctg atg acc ccg gtt ccg tgg tgC CAG TGG caa agg-5'
361/121                                      391/131
GGC CCT TAC TAC GGC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTT TCC TCA GCC
gly pro tyr tyr gly phe asp tyr trp gly gln gly thr thr leu thr val ser ser ala

421/141                                      451/151
AAA ACA ACA GCC cca tcg gtc tat cca ctC CCG GG
lys thr thr ala pro ser val tyr pro leu pro
```

Figure 27b. Sequences of the heavy chain PCR product amplified from SV5-P-k hybridoma cDNA.
The lower case in the DNA sequences indicates the primer-annealing regions, thus, although the primers specific for the leader sequences were degenerate, the sequence may not necessarily be genuine. PCR primers used for cloning of the variable regions into *E. coli* vectors expressing Fab and scFv fragments of antibody are shown above the sequences.

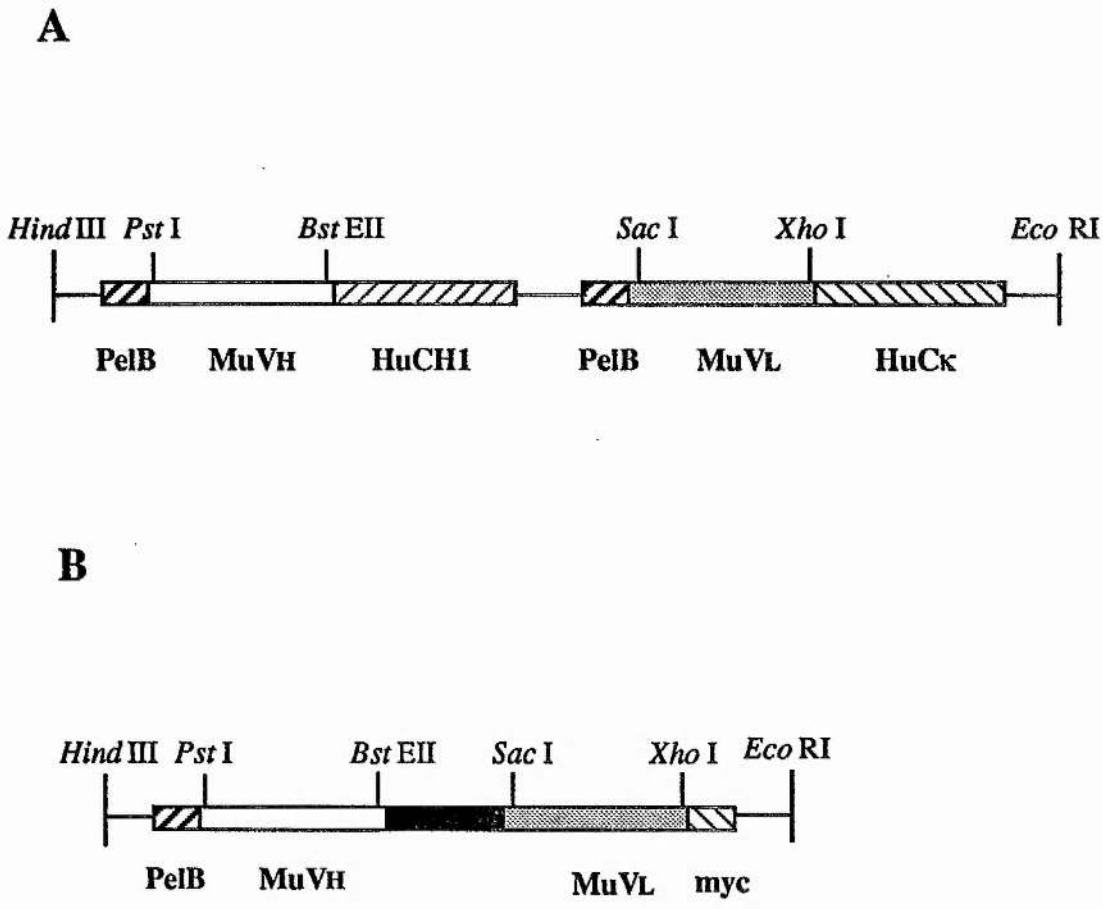


Figure 28. Constructs for expression of chimeric Fab and single chain Fv fragments of antibody in *E. coli*.
DNA carrying genes for expression of Fab (A) and scFv (B) antibody fragments in bacteria. The HuC_H1 and HuC_κ regions of the Fab fragment originated from human antibody genes. (B) In the scFv gene fragment, the heavy and light chain variable domains were connected by an oligoglycine linker. mAb epitope derived from the *myc* protein was attached to the C-terminus of the scFv fragment to facilitate its detection. The *Hind*III - *Eco*RI DNA fragments were in both cases inserted into the corresponding sites of the plasmid pUC119 polylinker. In addition to the pUC19 sequences, plasmid pUC119 contains an M13 origin of replication, and so its ssDNA can be synthetized and packaged into a helper phage. The expression of antibody fragments was under control of the *lacZ* promoter and could be induced by addition of IPTG. *Pelb* is a bacterial leader sequence enabling an efficient transport of nascent proteins into periplasm. The murine V genes of interest were inserted between the indicated restriction endonuclease sites.

ELISA, confirmed the fidelity of the isolated variable regions. Although the reaction of the chimeric Fab fragment yielded a much darker colour than that of the scFv, nothing could be inferred about the relative binding affinities of these fragments for the Pk tag oligopeptide. These remain to be determined.

D.2. Construction of eukaryotic vectors expressing engineered immunoglobulin chains.

D.2.1. Construction of genes for the reshaped V regions.

In the second stage of reshaping SV5-P-k mAb, complementarity-determining regions (CDRs) of human heavy and light chains were substituted with those derived from SV5-P-k variable regions, so that only about 9% of the total amino acid sequences were of a murine origin. This was achieved by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987). Briefly, three mutagenic kinased primers per variable region, each corresponding to one CDR (also see Fig. 4), were annealed to single-stranded human gene templates in M13, which contained incorporated uridine. The primers were extended *in vitro* by T7 DNA polymerase in the presence of T4 DNA ligase and the resulting double-stranded DNA species were transfected into *E. coli*. Because of the presence of uridine, the original templates in a wild-type bacteria were preferentially degraded, while the mutagenized strands generated *in vitro* were selectively replicated (also see Fig. 31).

D.2.1.1. Construction of gene for reshaped heavy chain V region.

The framework region of the human antibody KOL (Kabat *et al.*, 1987) inserted in vector M13KOLHuVH (K. Armour, unpublished data) was used as a template for engineering of the heavy chain. This M13-derived vector also carried approximately 260 bp upstream of the V_H gene, which contained the promoter region and two leader sequence exons (plus the intron), and 200 bp downstream of the V_H 3' splice-donor site (Fig. 29).

The reshaped V_H gene, designated rH, was generated using H1, H2 and H3 primers corresponding to CDR1, CDR2 and CDR3 sequences, respectively, for the *in*

Figure 29. Schematic structure of the inserts in M13KOLHuVH and M13VKPCR1 vectors.
The figure schematically depicts the *Hin*dIII-*Bam*HI fragments in M13-derived vectors used as templates for the oligonucleotide-directed mutagenesis. Dark boxes represent the leader sequence- and the empty box variable region-coding sequences. 'V' indicates splice sites.

vitro synthesis of the second strand. Primer H2 also mutated AGC codon 75 for serine to proline codon, CCC. After transformation of bacteria with the *in vitro* extension reaction mixture, one out of eight sequenced M13 clones contained grafted CDR1, CDR3 and the proline codon, but not CDR2. Because repeated attempts to graft CDR2 using the H2 primer were abortive, a second primer, termed H2.2, was designed, which was shorter at the 5'-end and extended at the 3'-end. This primer successfully substituted the DNA corresponding to KOL CDR2 with that of SV5-P-k.

In addition to the grafted CDR, the reshaped heavy chain contained two substitutions in the framework, Ser75->Pro and Arg98->Ser (see also Table 5). These changes had been suggested by B. Harris (Scotgen, Aberdeen) in order to improve the binding affinity of the reshaped variable domain. As can be seen in Fig. 30, the reshaped heavy chain had two 'accidental' nucleotide/amino acid substitutions just upstream of CDR1, but these were assumed to be unimportant for the overall structure of the variable domain.

D.2.1.2. Construction of gene for reshaped light chain V region.

The SV5-P-k light chain CDRs were grafted onto the framework region of the human myeloma protein REI (Reichman *et al.*, 1988) inserted in vector M13VKPCR1 (Orlandi *et al.*, 1989). This vector carried approximately 190 bp upstream sequences of the V_κ gene, which contained the promoter region and two leader sequence exons (plus the intron), and 40 bp of the 3'-end and the 3' non-coding sequences taken from human J_κ region sequences (Reichman *et al.*, 1988; Fig. 29). After *in vitro* synthesis

Reshaped heavy-chain V-region gene (rH)

```
1/1                                                 31/11
gag gtc caa ctg gtg gag agc ggt gga ggt gtt gtg caa cct ggc cgg tcc ctg cgc ctg
glu val gln leu val glu ser gly gly gly val val gln pro gly arg ser leu arg leu
61/21                                               91/31
tcc tgc tcc tcg tct ggc tat acc ttc acc AGT TTT GGA ATG CAC tgg gtg aga cag gca
ser cys ser ser ser gly tyr thr phe thr ser phe gly met his trp val arg gln ala
121/41                                              151/51
cct gga aaa ggt ctt gag tgg gtt gca TAC ATT AAT ACT GAC AGT ACT ACC ATC TAC TAT
pro gly lys gly leu glu trp val ala tyr ile asn thr asp ser thr thr ile tyr tyr
181/61                                              211/71
GGA GAC ACA GTG AAG GGC aga ttt aca ata tcg cga gac aac CCc aag aac aca ttg ttc
gly asp thr val lys gly arg phe thr ile ser arg asp asn pro lys asn thr leu phe
241/81                                              271/91
ctg caa atg gac agc ctg aga ccc gaa gac acc ggg gtc tat ttt tgt gca agc GCG GGC
leu gln met asp ser leu arg pro glu asp thr gly val tyr phe cys ala ser ala gly
301/101                                             331/111
CCT TAC TAC GGC TTT GAC TAC tgg ggc caa ggg acc ccg gtc acc gtc tcc tca
pro tyr tyr gly phe asp tyr trp gly gln gly thr pro val thr val ser ser
```

Reshaped light-chain V-region gene (rL)

```
1/1                                                 31/11
gac atc cag Ctg acc cag agc cca agc agc ctg agc gcc agc gtg ggt gac aga gtg acc
asp ile gln leu thr gln ser pro ser ser leu ser ala ser val gly asp arg val thr
61/21                                               91/31
atc acc tgt AGG TCT AGT AAG AGT CTC GTA CAT AGT AAT GGC ATC ACT TAT TTG TAT tgg
ile thr cys arg ser ser lys ser leu val his ser asn gly ile thr tyr leu tyr trp
121/41                                              151/51
tac cag cag aag cca ggt aag gct cca aag ctg ctg atc tac CAG ATG TCC AGC CTT GCC
tyr gln gln lys pro gly lys ala pro lys leu leu ile tyr gln met ser ser leu ala
181/61                                              211/71
TCA ggt gtg cca agc aga ttc agc ggt agc ggt agc ggt acc gac ttc acc ttc acc atc
ser gly val pro ser arg phe ser gly ser gly ser gly thr asp phe thr phe thr ile
241/81                                              271/91
agc agC Ctc cag cca gag gac atc gcc acc tac tac tgc GGT CAA ATT CTA GAA CTT CCA
ser ser leu gln pro glu asp ile ala thr tyr tyr cys gly gln ile leu glu leu pro
301/101                                             331/111
TTC ACG ttc ggc caa ggg acc aag gtg gTG atc aaa cgt GAG TAG AAT TTA AAC TTT GCT
phe thr phe gly gln gly thr lys val val ile lys arg glu AMB asn leu asn phe ala
361/121
TCC TCA GTT GGA TCC
ser ser val gly ser
```

Figure 30. Sequences of reshaped rH and rL variable regions.
The figure shows DNA and deduced protein sequences of reshaped variable regions. Nucleotides in the variable regions that differ from the original human chain genes are shown in capitals.

of the second strand using the L1, L2 and L3 mutagenic primers and transformation, one M13 clone, out of twelve sequenced, contained all three murine CDRs.

D.2.2. Construction of chimeric V region genes.

Chimeric heavy (cH) and light (cL) chains were also constructed, which consisted of variable murine domains joined to constant human domains. Because chimeric Fab expressed in *E. coli* readily recognized the Pk tag, it was expected that the whole chimeric antibody (cLcH) would behave similarly. Furthermore, affinity comparison of combined chimeric light and reshaped heavy (cLrH), and reshaped light and chimeric heavy (rLcH) chains to the whole chimeric antibody (cLcH) should facilitate the identification of possible binding problems to reshaped heavy, light or both chains.

D.2.2.1. Construction of chimeric heavy V gene.

For the construction of the chimeric heavy chain (cH), the whole murine V_H region gene was inserted into the M13KOLHuVH for addition of the human V_H gene-flanking sequences (Fig. 29). To do that, a *Pst*I site was generated at the 5'-end of the KOL V_H gene using H-Pst primer and oligonucleotide-directed mutagenesis. The murine V_H gene was then PCR amplified using the same primers as used for the cloning of the murine V_H into the Fab and scFv vectors (Fig. 27b). The PCR product was digested with *Pst*I and *Bst*EII restriction endonucleases and used to substitute for the KOL gene in *Pst*I- and *Bst*EII-digested double-stranded replicative form (RF) of bacteriophage M13KOLHuVH.

D.2.2.2. Construction of chimeric light V gene.

A chimeric light chain (cL) gene was generated by PCR amplification of the murine V_K using primers L-PvuBACK and L-BamFOR, digesting the PCR product with *Pvu*II and *Bam*HI enzymes and inserting it between the same sites in the RF of M13VKPCR1. The L-BamFOR primer included DNA sequences beyond the ORF of V_K including the necessary 3' splice-donor site (Reichmann *et al.*, 1988; Fig. 29).

Table 5. Recombinant heavy and light chains derived from SV5-P-k.

cL	chimeric light chain (murine variable/human constant regions)
rL	straight CDR graft
cH	chimeric heavy chain (murine variable/human constant regions)
rH	CDR graft + substitutions of Ser75->Pro and Arg98->Ser

D.2.3. Assembly of complete immunoglobulin genes in eukaryotic expression vectors.

In the next step of the construction of recombinant immunoglobulin genes, the reshaped and chimeric variable regions (Table 5) were coupled to human IgG1 and human kappa constant regions for expression in eukaryotic expression vectors.

Eukaryotic vectors expressing complete cH and rH heavy chains were constructed as follows. The reshaped and chimeric V_H genes together with the appropriate flanking DNA sequences were cut out from the RF of appropriate bacteriophage DNAs using *Hin*dIII and *Bam*HI sites and cloned into eukaryotic expression vector pSVgpt (Mulligan and Berg, 1981). The gene coding for human IgG1 heavy chain (Takahashi *et al.*, 1982) was then transferred from plasmid pBSG18-HuIgG1 into pSVgptH vectors using *Bam*HI sites.

cL and rL light chains were expressed using pSVhyg vector (Orlandi *et al.*, 1989). Both the chimeric and reshaped V_K genes flanked by appropriated DNA sequences were excised from the M13 vectors and inserted into pSVhyg using *Hin*dIII and *Bam*HI sites. As an illustration of the steps involved in the construction and assembly of the complete immunoglobulin genes, the cloning of the rL light chain is schematically depicted in Fig. 31.

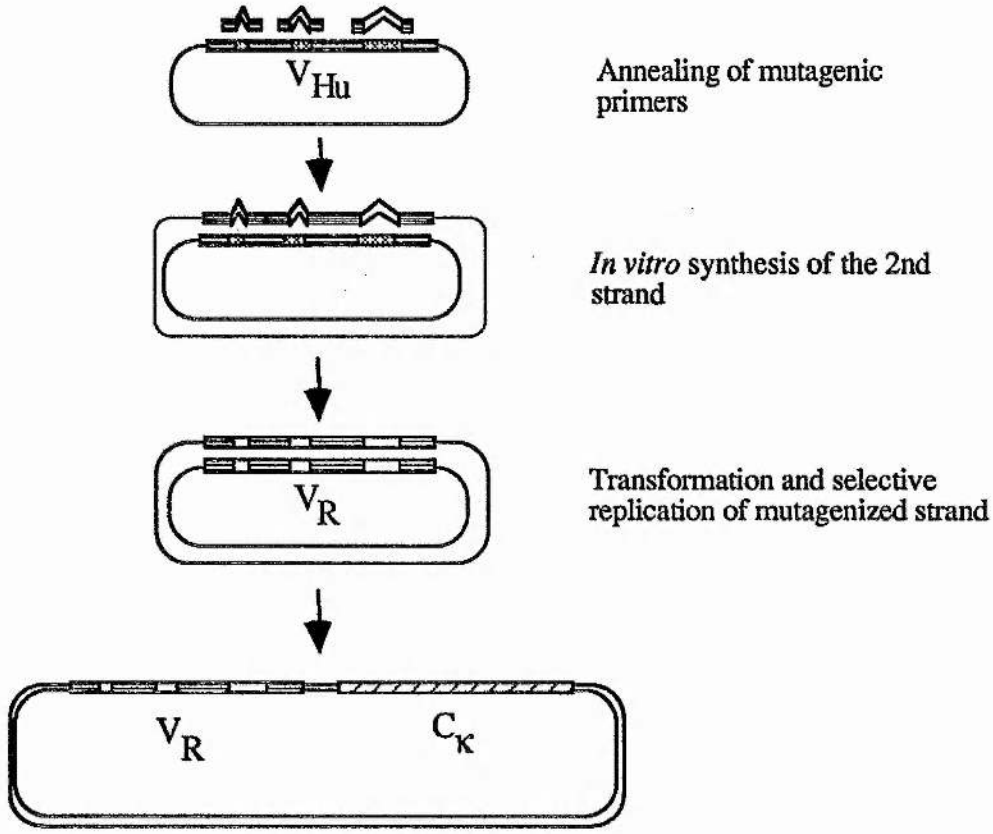


Figure 31. Construction and assembly of the rL light chain gene.
(The schematics does not show the leader sequence and other V gene flanking regions described in Fig. 29.)

D.3. Generation of stably transfected CHO cell clones expressing recombinant immunoglobulin chains.

Vectors pSVhygL and pSVgptH expressing the light and heavy chains under control of immunoglobulin promotors were transfected in Chinese hamster ovary (CHO) cells and the successful transfectants were selected by their ability to grow in the presence of hygromycin B (pSVhygL) and mycophenolic acid plus xanthine (pSVgptH). Hygromycin B inhibits protein synthesis by disrupting translocation and promoting mistranslation. The hygromycin B-phosphotransferase gene product

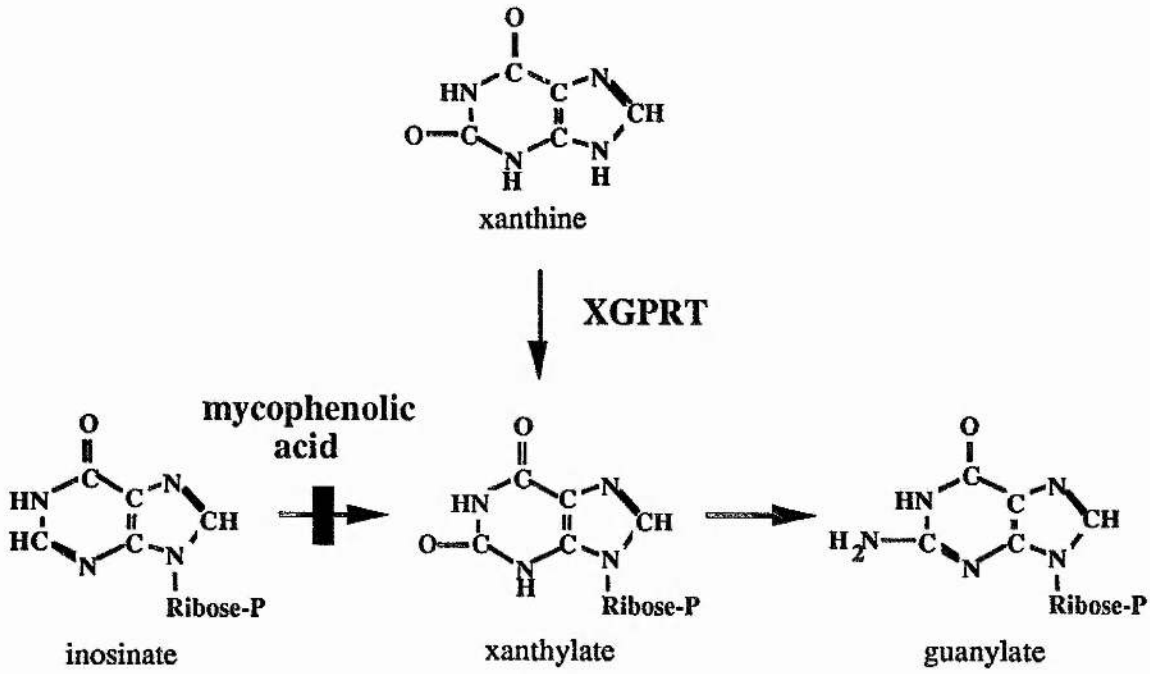


Figure 32. Principle of the metabolic selection for XGPRT-expressing cells by mycophenolic acid/xanthine.

detoxifies hygromycin B by phosphorylation (Gritz and Davies, 1983). Mycophenolic acid blocks the pathway for *de novo* synthesis of guanylate (GMP) and the expression of xanthine-guanine phosphoribosyltransferase (XGPRT or gpt), an enzyme normally absent in mammalian cells, allows transfectants to produce guanylate from xanthine (Mulligan and Berg, 1981). The relevant metabolic pathways of the mycophenolic acid selection are depicted in Fig. 32.

Firstly, pSVhygL vectors expressing either cL or rL light chains were transfected into CHO cells using electroporation and the transformants were selected for growth in the presence of hygromycin B. Nine individual colonies of each of pSVhygcL- and pSVhygrL-transfected cells, designated CHO-cL and CHO-rL, respectively, were ring-cloned and expanded. The presence of light chains was tested on a dot blot in both the cell lysates and tissue culture supernatants using HRP-conjugated antibodies specific for the human κ light chain followed by ECL. Fig. 33 shows a dot blot analysis of the CHO-rL clones. As judged by the cell lysates, of the

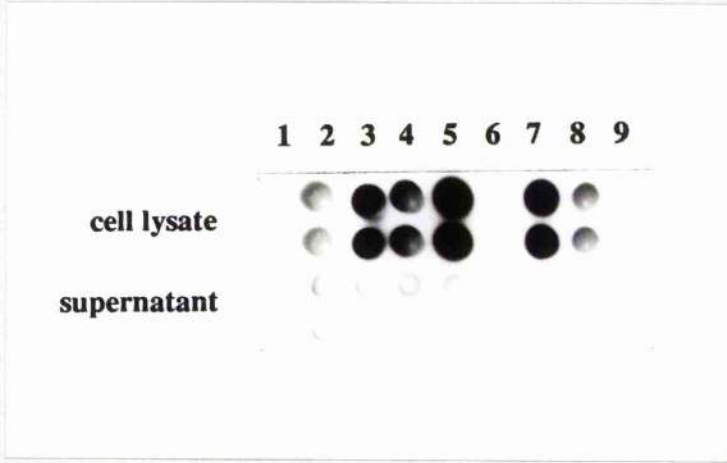


Figure 33. Expression of the rL light chain by CHO-rL cell clones.
Nine clones of pSVhygrL-transfected CHO cells for expression of the rL light chain. Cell lysates (top) or tissue culture supernatants (bottom) were dotted on a nitrocellulose filter and the filter was probed using HRP-conjugated anti-human κ light chain mAb followed by ECL. The figure shows a 20-min. exposure.

nine hygromycin B-resistant clones, two did not express any light chain at all and the expression levels in the others varied. No significant amounts of the rL light chain were detected in the supernatants.

The CHO-cL and CHO-rL clones producing the highest levels of the respective light chains were transfected with vectors pSVgptcH or pSVgptrH, so that cells expressing all the four possible combinations of heavy and light chains were generated. Transformats were selected by growth in the presence of hygromycin B and mycophenolic acid/xanthine, ring-cloned and are being currently grown to sufficient cell numbers to allow testing for the expression of both the heavy (using HRP-conjugated protein A) and light (as above) chains. Recombinant immunoglobulin chains produced by 'double-positive' clones will be tested for the chain association and binding specificity in ELISA.

DISCUSSION

1. Pk tag for assembly of SMAA vaccines.

The binding of monoclonal antibody SV5-P-k to the P protein of simian virus 5 was successfully competed with a nonapeptide (Southern *et al.*, 1991). This nonapeptide, extended by 5 adjacent amino acids from the P protein and designated Pk, had a number of properties, which made it attractive for the construction of vaccines. It retained its antigenicity when coupled to the C-terminus of larger proteins. This was initially demonstrated using the nucleocapsid protein p27 of simian immunodeficiency virus both denatured (as shown by a Western blotting in Fig. 11) and in a native conformation (as shown by capture and assembly of p27-Pk into SMAA complexes in Fig. 10). This finding was subsequently extended for other non-glycosylated SIV proteins (e.g. Figs. 14 and 17). The Pk tag also remained antigenic when attached to the C-terminus of a glycoprotein, SIV gp160, (Fig. 25) and, in that position, did not interfere with transport and post-translational modification/ glycosylation of the gp160 glycoprotein in insect cells. Although this finding may be limited to this particular situation, it suggests that the Pk tag may be a valuable universal affinity tag for the purification of glycoproteins. Furthermore, the Pk tag does not seem to interfere with the transport of proteins across the plasma membrane and cellular wall of *E. coli*. This was demonstrated by a successful secretion of the Fv antibody fragment with the Pk tag attached to the C-terminus of its C_H1 domain from bacteria (T. H. and G. Winter, unpublished observation). In the past, this had not been the case for all Fv terminal tags (G. Winter, personal communication). This finding

may be of significance for the large-scale production of Pk tag-linked antigens. The secretion of antigens from bacteria may facilitate immensely the industrial purification of antigens and, thus, may lead to more economical methods of vaccine production. It is also important to minimize the immune responses directed against the non-antigen parts of the vaccine. In this respect, the Pk tag was only weakly immunogenic when bound to SV5-P-k mAb in the SMAA complexes (Fig. 12 and Randall *et al.*, 1993a).

2. Two-tag purification of proteins.

Three commercially available expression vectors pGEX-2T, pQE-9 and pVL1393 were modified (Fig. 34) and used to express SIV proteins with the Pk tag at their C-termini. The non-glycosylated SIV proteins also had either glutathione *S*-transferase (GST) or histidine (His) affinity tags attached to their N-termini. A general purification method was developed, in which the recombinant proteins were first purified via their N-terminal His tag on a nickel-affinity column and then via their C-terminal Pk tag using SV5-P-k mAb (Fig. 20). Despite lower than 10 $\mu g.l^{-1}$ levels of expression for some SIV proteins (Table 4), analytical quantities of all the His-SIV-Pk proteins using a two-tag purification protocol were prepared (Fig. 21).

The number of proteins that need to be produced and isolated to a high degree of homogeneity increases every day. Purification involving two different tags, each attached to one end of a recombinant protein, represents a simple and rapid technique for isolation of full-size proteins (Fig. 35). This strategy may be particularly useful for the large-scale production of immunogens needed in the construction of subunit vaccines. There is an increasing requirement for precisely defined components of vaccines and two-tag purification provides proteins that are intact at both their N-and C-termini. However, it should be noted, that even protocols involving tags attached to both protein termini occasionally co-purify partially degraded species due to protein

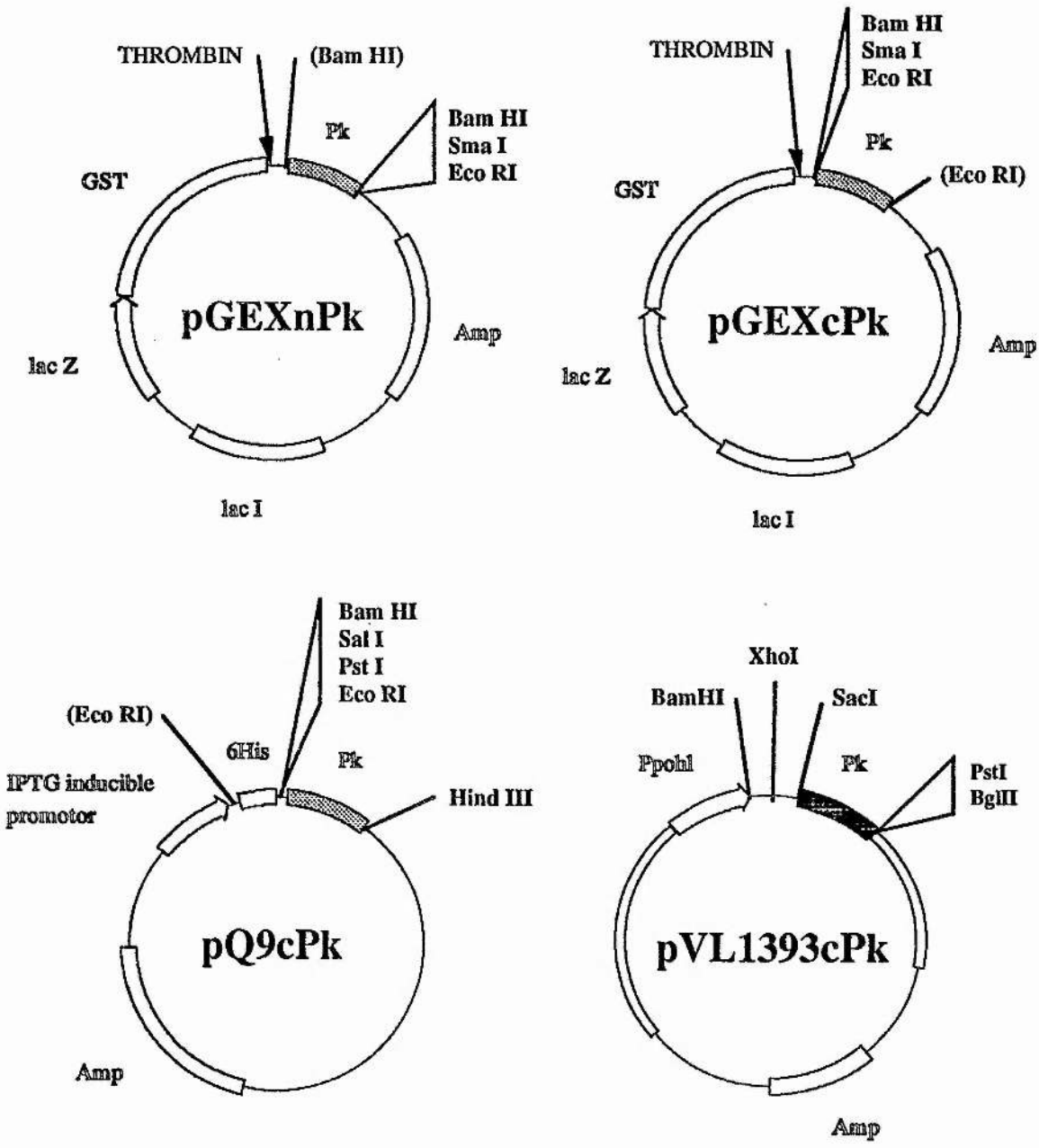


Figure 34. DNA vectors for expression of tagged proteins.
Vectors pGEXnPk, pGEXcPk and pQ9cPk are designed for a single-cloning-step addition of two purification tails to bacterially expressed proteins. pVL1393cPk is a transfer vector for the construction of recombinant baculoviruses.

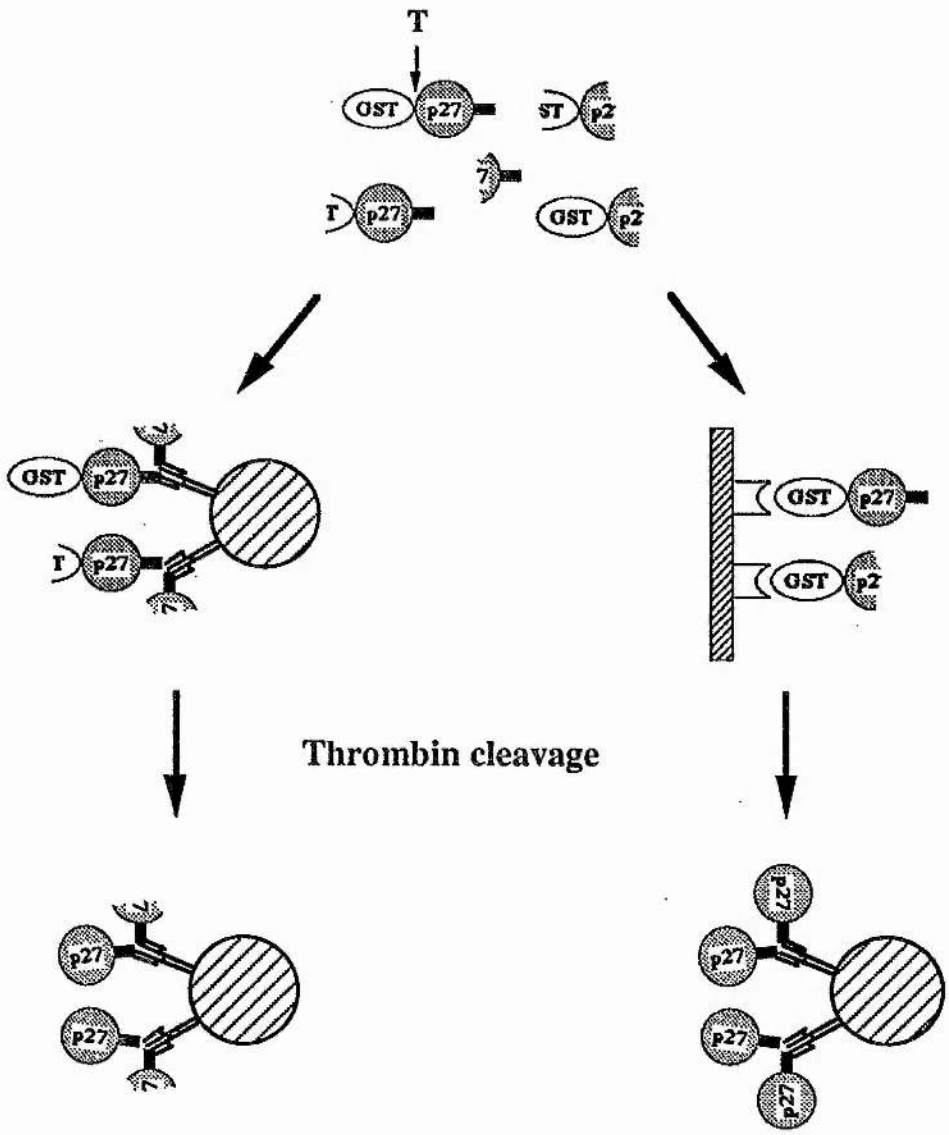


Figure 35. Schematics of a two-step purification of SIV 27-Pk expressed from vector pGEX27Pk.
The use of two affinity tags ensures purification of full-size proteins (right).
T indicates the site of thrombin cleavage.

aggregation or on-going proteolytic degradation during the purification procedure. Furthermore, two-step purification is less prone to contamination than protocols attempting to purify the protein of interest in a single step. This is especially relevant for antigens that are difficult to express at high levels in host organisms.

Short tags may also have advantage of low immunogenicity and may not be necessary to remove from recombinant proteins, which are intended for use as immunogens. As has been demonstrated, both the Pk (Fig. 12) and His (Randall *et al.*, 1993a) tags induce only weak antibody responses. However, it is difficult to predict whether small tags may contain immunodominant T cell sites and thus interfere with the induction of cell-mediated immunity. If this is the case, it may be possible to reduce the tag size. Indeed, an antigenic sequence as short as three amino acid residues has been identified (R.E. Randall, personal communication). Also, a chelating N-terminal dipeptide HisTrp was sufficient to purify proinsulin (11 amino acids; Smith *et al.*, 1988). In this case, the binding of HisTrp-proinsulin to an immobilized Ni^{2+} was probably partially facilitated by the presence of two histidine residues in proinsulin. Thus, the use of HisTrp tag and nickel-chelating chromatography was a sensible choice of a purification strategy, which suggested that even using 'universal' affinity tags, each protein may have to be approached individually to select an optimal purification procedure. Thus, one may envisage a situation, in which two amino acid residues at the N-terminus and three amino acids at the C-terminus may allow a two-step purification of full-size proteins.

3. Levels of protein expression in prokaryotic cells.

Purification protocols involving terminal Pk and His tags worked well for all the proteins tested. The major obstacle for the large-scale protein preparations of a number of SIV proteins was their level of expression. While the yields of His-17-Pk approached 5 - 10 mg per litre of induced culture (approximately 2.5 g of wet bacterial pellet), proteins such as His-Npol^{i}-Pk and His-prot^{i}-Pk were produced in amounts less than 10 $\mu g.l^{-1}$. For each of the SIV protein, time-course of expression

(Fig. 18) and IPTG-induction dose-response experiments at two different temperatures (Fig. 19) were carried out, which, however, resulted in only marginal increases in expression levels and only for some proteins. Increased protein yields may also result from monitoring the physiology of bacteria during the period of IPTG induction of protein expression, a factor that was not examined in this work. Among the parameters to measure would have been the level of nitrogen in the culture medium, the accelerating rate of consumption of initial glucose or the molar fraction of CO_2 and O_2 in the exit gas (Pack *et al.*, 1993).

Large differences in the relative levels of expression between SIV genes were observed in both pGEXcPk and pQ9cPk vectors. Thus, the problems appeared to be associated with the particular DNA sequences or polypeptide chains expressed rather than being dependent on the expression vector system used. There are several possible explanations for this observation. An obvious problem in the overproduction of proteins in prokaryotic cells is proteolytic degradation. It can be envisaged, that foreign proteins would be rapidly degraded, if their conformation or amino acid sequence did not offer protection from the host intracellular proteases. Moreover, some proteins, e.g. those carrying regulatory functions, may be inherently unstable. There is a number of ways how to deal with protein instability. One possibility is to use *E. coli* strains deficient in certain proteases or other strains carrying the so called *pin* (for protease inhibitor) gene of bacteriophage T4 (Simon *et al.*, 1983). Alternatively, recombinant proteins may be secreted from bacteria into the growth medium, where proteolytic enzymes may be different and/or their levels reduced.

As already mentioned above, proper folding influences protein stability in the cell. The proper folding of recombinant proteins in bacteria may be facilitated by induction of bacterial cultures at lower temperatures, which probably slows the overall translation rate and gives the translated protein time to assume its tertiary conformation. Codon usage and distribution in the gene may also affect protein folding. It was suggested by Purvis and collegues (1987), that a set of rare codons may cause a translational pause at key junctures in protein biosynthesis, which may, in turn, allow a discrete domain to fold more efficiently. This fine co-translational

regulation may be disturbed in hosts, which differ in codon usage from the gene's original species. Moreover, high proportion or accumulation of infrequently used codons in a gene may cause a limitation of amino acid-charged tRNAs, which may significantly increase the frequency of mistranslations or decrease the overall protein production (Robinson *et al.*, 1984). The only potential problem-causing codons of mammalian genes expressed in prokaryotes are the AGG and AGA codons for Arg. However, when the SIV genes were searched for these codons, there was no immediate difference in their usage between well and poorly expressed His-SIV-Pk proteins.

Many different post-translational modifications of proteins have been described in eukaryotes including glycosylation, phosphorylation, acetylation, amidation, sulphation, attachment of fatty acids and formation of unusual amino acids, none of which, as far as it is known, occurs in bacteria. These modifications may again influence the protein conformation and contribute to protein stability.

Cell toxicity of His-SIV-Pk proteins expressed in bacteria should be also considered. This could be partially overcome by a more tightly-controlled inducible promotor than the *lac*-based system operating in pGEX-2T and pQE-9 plasmids. An example of a tight expression control is the T7-*lac* double repression system (Studier *et al.*, 1993). In these constructs, a *lac* operator is placed between a T7 promotor and the gene of interest. The transcription of the T7 polymerase gene in the same bacterium is also controlled by *lac* operator. Thus, in the presence of *lac* repressor, both transcription of the T7 polymerase gene by *E. coli* RNA polymerase and that of the target gene by T7 polymerase are inhibited. Their normal transcription can be restored by addition of IPTG. Alternatively, bacteria could be induced at higher temperature than 37 °C, which results in sequestering of proteins into inclusion bodies (J. Sedlacek, personal communication). Also, the use of non-bacterial hosts such as baculovirus vectors or yeasts is a possibility always worth pursuing.

Finally, mRNA secondary structures may account for the differences seen in expression of different SIV proteins from identical plasmids. In particular, the Shine-Dalgarno (30S ribosomal subunit-binding site) and more so the AUG sequences have

to be readily accessible for an efficient translation, i.e. should not be a part of dsRNA in secondary mRNA structures (Roberts *et al.*, 1979). For the same reasons, reconstruction of the 5'-end of expressed gene has been recommended, that maximizes the A+T content, but preserves amino acid sequence (DeLamarter *et al.*, 1985).

In essence, there is room for increasing of the levels of SIV protein expression, but, as it is with the traditional protocols of protein purification, there is not a straightforward method to follow. There are two ways how to pursue the low expression levels. The first approach is to take the *His-SIV-Pk* genes one by one and identify in each case the particular problem. Then, according to both the theoretical and empirical principals, steps may be taken in attempt to increase their levels of expression. Alternatively, bacteria may be exposed to a mutagen and searched for high-expression mutants. Both these approaches are labour- and time-intensive and are beyond the scope of this thesis.

4. Antigenicities and immunogenicities of recombinant SIV proteins.

One of the reasons for expressing and purifying all of the SIV proteins was to produce reagents for immunization/protection experiments in macaques. The idea was to prepare SMAA complexes containing all the SIV antigens and use these for vaccination of experimental animals, which would be subsequently challenged with SIV. However, before the SMAA complexes containing SIV proteins were used in monkeys, their immunologic fidelities, i.e. their abilities to induce antibodies and cell-mediated immune responses, which could recognize authentic SIV antigens or SIV-derived peptides, had been evaluated in smaller and cheaper animals such as mice.

4.1. Bacterially produced non-glycosylated SIV proteins.

All the so-far tested recombinant non-glycosylated SIV proteins assembled into SMAA complexes and used for immunizations of mice (His-p17-Pk, His-p27-Pk, His-rt-Pk, His-vpr-Pk, His-vpr-Pk) induced antibodies, which readily recognized

authentic Western-blotted proteins in SIV-infected cell lysates (Fig. 13 and Randall *et al.*, 1993a, 1993b).

Western blotted recombinant SIV proteins were also employed in the analysis of the antibody responses in SIV-infected macaques. Using HRP-conjugated protein A followed by ECL, clear humoral responses to endonuclease, p15, p17, p27, rt and vif as well as weak responses against vpx and protease were observed (Fig. 22). No antibodies specific for nef, rev, tat or vpr were detected. Although some monkeys have previously produced nef-specific humoral responses (M. Cranage, personal communication), the negative results in this experiment suggest, when translated to humans, that it may be difficult to use antibody responses to nef to distinguish between infection with wild-type HIV and any administered attenuated nef-deleted virus vaccines. However, the possible reasons for a negative signal in this type of assay were several: (i) the antibodies were not present in the monkey serum, a genuinely negative results; (ii) the antibodies were present, but not detected, because they were specific for structural epitopes on the non-denatured proteins and failed to recognize the denatured polypeptides; alternatively, (iii) cross-reacting antibodies present in the serum, which were generated by linear epitopes on authentic SIV proteins, failed to recognize the same stretch of amino acid residues on recombinant proteins, i.e. the recombinant SIV proteins did not have the desired fidelity, a situation which is difficult to picture unless some post-translational modifications had occurred on either recombinant or mammalian cell-produced SIV proteins; finally, (iv) antibodies in the monkey serum did bind the Western blotted proteins, but failed to bind the HRP-conjugated protein A.

The ability to induce lymphoproliferative responses has been examined using His-rt-Pk (A. Green, personal communication). After immunization with SMAA complexes containing His-rt-Pk, murine splenocytes were removed and incubated with the recombinant antigens. Incorporation of radiolabel was observed after *in vitro* stimulation with His-rt-Pk, but not His-p17-Pk, which indicated, that the lymphoproliferative responses were specific for the SIV reverse transcriptase rather than for the terminal His and Pk tags.

4.2. SIV env produced in insect cells.

Serum from SIV-infected macaques recognized efficiently Western-blotted recombinant gp160-Pk (Fig. 25, panel D, lanes 1 and 2), thus the insect cell-produced gp160-Pk was antigenicly appropriate. In order to evaluate the ability of recombinant gp160-Pk to induce antibody responses capable of recognition of the authetic SIV envelope glycoprotein, mice were immunized with gp160-Pk-containing SMAA complexes. Vigorous humoral responses were generated, that readily recognized recombinant gp160-Pk. However, the murine serum reacted only weakly with the Western-blotted authentic gp160 monomer in SIV-infected mammalian cells (Fig. 25, panel E, lane 3). If the slower-migrating band on the Western blot of SIV-infected mammalian cell lysates was indeed a gp160 dimer, as argued earlier, then the antibodies induced by immunization recognized the glycoprotein preferentially in a dimeric rather than monomeric form.

4.3. Protection experiments in macaque monkeys.

As an extension of this work, the preparation of large-enough quantities of these SMAA complexes for protection experiments in macaques was attempted. However, because of problems with the levels of protein expression in *E. coli*, preparation of only six SIV antigens have been successfully achieved to-date (C. Botting and D. Young, personal communication). Thus, only alum-antibody-antigen complexes of His-17-Pk, His-27-Pk, His-rt-Pk, His-vpr-Pk, His-vpx-Pk (prepared by eluting the antibody-antigen complexes from protein A-sepharose and subsequent precipitation of the complexes into alum), and *S. aureus*-antibody-gp160-Pk complexes have been used for macaque immunizations. In addition to the limited number of antigens, there are several potential factors complicating the interpretation of a possible negative outcome of this protection experiment. Firstly, all the non-glycosylated SIV proteins used for the first protection experiment were derived from SIV molecular clone BK28, the precise derivation of which is difficult to trace (Fig. 7) and which is not widely used. Consequently, the macaques will be challenged with molecular clone SIVmac251(32H)(J5), a virus homologous to the gp160-Pk, but heterologous for the non-glycosylated antigens. Secondly, there has not

been a single case reported of protection of macaques against SIVmac infection using purified recombinant antigens as the only immunogens (Stott, 1993). This may be, however, partially due to the fact, that the immunization studies predominantly concentrated on the SIV *env* and *gag* gene products. Thirdly, alum gels were shown by Shearer and Clerici (1993) to preferentially induce the Th2 subset of T helper cells, which leads to induction of humoral responses rather than CTL, and, in the past, antibodies did not seem to correlate with macaque protection. Finally, if the antibody component of SMAA complexes plays a role in the induction of immune responses, then it is not clear, how well the murine IgG2a antibody interacts with the simian immune system. Thus, a negative result of the protection experiment will have little meaning for the evaluation of immunogenic potentials of the involved recombinant SIV proteins. On the other hand, a positive outcome of these experiments would allow dissecting of the protective immune responses against SIV infection in macaques and would enable the correlates of protection to be established.

5. Areas of future investigation.

5.1. The nature of solid matrix.

One component of SMAA complexes, that has not been addressed in this work, is the nature of the solid matrix. It is clearly an area of SMAA complex development, which will require future attention. It may be advantageous, if the solid matrix were of a (i) particulate nature, which would facilitate preparation of the vaccine especially at the stage of the antigen purification. The (ii) size of particulate solid matrixes should be optimized, as it may influence the efficiency of capture of SMAA complexes by antigen-presenting cells and thus may influence the immunogenicity of SMAA complexes. The solid matrix should also be made of a (ii) biodegradable material, which would allow complete clearance of the matrix from the organism. Some materials used for incorporation of antigens or antigen-antibody complexes may allow (iii) sustained or timed release of the immunogen. Finally, the solid matrix should be (iv) inexpensive.

5.2. The nature of linkage between antigens and solid matrix.

The availability of cloned variable regions of the tag-specific mAb provides a unique opportunity to address the issue of importance of the antibody component of SMAA complexes in the immunogenicity of this type of vaccine. For example, different immunoglobulin isotypes can be used for the construction, of SMAA complexes, some of which do not bind complement or possess weak affinity for a certain class of Fc receptor. These experiments may also shed some light on which antigen-presenting cells actually process and present SMAA complex-delivered antigens. Alternatively, Fab or Fv fragments of antibodies, linked (e.g. via disulphide bonds) to particulate beads, may be used for the construction of SMAA complexes.

The antibody component may not be included in the vaccine design at all. Attaching tag-linked antigens by a non-covalent, affinity tag-mediated interaction directly to beads for vaccination purposes remains to be an attractive possibility. Such a strategy would retain the benefits of simplicity, universality and antigen purification discussed above for the solid matrix-antibody-antigen complexes, yet it would reduce the cost and chance of adverse immune reactions.

5.3. Increasing the immunogenicity of SMAA complexes.

As it was shown by Randall and co-workers (1991), immunization of mice with SMAA complexes was less efficient in inducing MHC class I-restricted CTL than infection of mice with virus. Thus, possibilities of increasing the immunogenic potency of SMAA complexes for induction of CTL should be explored. In general, proteins of immunostimulating properties, such as the cholera toxin subunit B or the subunit B from the heat-labile *E. coli* toxin, may be incorporated into SMAA complexes. Also, antigens may be included, which would target SMAA complexes to particular antigen-presenting cells or selectively stimulate certain aspects of immunity, i.e. mucosal immune responses.

6. SMAA complexes as potential human vaccines.

Monoclonal antibody SV5-P-k has been reshaped as a part of the development of SMAA complexes for potential use in humans. Humanization should improve the antibody's interaction with the human immune system and minimize its immunogenicity in the human milieu. In general, every antibody induces a certain level of anti-idiotypic antibodies, in part due to somatic mutations during affinity maturation. Thus it may not be surprising, that even reshaped antibodies used in a recent clinical trial retained some degree of immunogenicity (Isaacs *et al.*, 1992). Obviously, the fewer substitutions in the V region frameworks of the humanized antibody, the better. Every amino acid change introduced *in vitro* has not been seen, and therefore 'permitted', by the acceptor's immune system.

The work presented here has demonstrated, that, in general, the assembly of tag-based SMAA complexes is indeed a feasible way of constructing multivalent subunit vaccines. Taking advantage of this simple approach, multiple antigens, including a glycoprotein, have been assembled into SMAA complexes and used as immunogens. The cost of production of SMAA complex vaccines should not be prohibitive, providing that optimal expression systems for all the protein components are found. As for the cost of the antibody, mAbs can be produced at 100-mg to 1-g quantities per litre of tissue culture supernatant (R.E. Randall, personal communication). In conclusion, a universal vaccine core can be envisaged, which consists of humanized tag-specific monoclonal antibody attached to a suitable solid matrix, to which tag-linked antigens of choice are bound and used for vaccination.

REFERENCES

ACHA-ORBEA, H., SHAKHOV, A.N., SCARPELLINO, L., KOLB, E., MULLER, V., VESSAS-SHAW, A., FUCHS, R., BLOCHLINGER, K., ROLLINI, P., BILLOTE, J., *et al.* (1991). Clonal deletion of Vβ14-bearing T cells in mice transgenic for mammary tumour virus. *Nature* **350**, 207-211.

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.

AGY, M.B., FRUMKIN, L.R., COREY, L., COOMBS, R.W., WOLINSKI, S.M., KOEHLER, J., MORTON, W.R., AND KATZE, M.G. (1992). Infection of *Macaca nemestrina* by human immunodeficiency virus type 1. *Science* **257**, 103-106.

AHLUWALIA, N., BERGERON, J.J.M., WADA, I., DEGEN, E., AND WILLIAMS, D.B. (1992). The p88 molecular chaperon is identical to the endoplasmic reticulum protein, calnexin. *J. Biol. Chem.*. **267**, 10914-10918.

ALCAMI. A., AND SMITH, G.L. (1992). A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* **71**, 153-167.

ALLAN, J.E., AND DOHERTY, P.C. (1985). Consequences of a single Ir-gene defect for the pathogenesis of lymphocytic choriomeningitis. *Immunogenetics* **21**, 581-590.

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

AMEISEN, J.-C., AND CAPRON, A. (1991). Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. *Immunol. Today* **12**, 102-105.

AMIT, A.G., MARIUZZA, R.A., PHILLIPS, S.E.V., AND POLJAK, R.J. (1986). 3-dimentional structure of an antigen-antibody complex at 2.8-Å resolution. *Science* **233**, 747-753.

ANDERSON, K., CRESSWELL, P., GAMMON, M., HERMES, J., WILLIAMSON, A., AND ZWEERINK, H. (1991). Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen-processing mutant cells to class I-restricted cell-mediated lysis. *J. Exp. Med.* **174**, 489-492.

ARNOLD, D., DRISCOLL, J., ANDROLEWITZ, M., HUGHES, E., CRESSWELL, P., AND SPIES, T. (1992). Proteasome subunits encoded in the MHC are not generally required for the processing of peptides bound to MHC class I molecules. *Nature* **360**, 171-173.

ARTHUR, L.O., BESS, J.W., SOWDER, R.C., BENVENISTE, R.E., MANN, D.L., CHERMANN, J.-C., AND HENDERSON, L.E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* **258**, 1935-1938.

ASHORN, P., MOSS, B., WEINSTEIN, J.N., CHAUDHARY, V.K., FITZGERALD, D.J., PASTAN, I., AND BERGER, E.A. (1990). Elimination of infectious human immunodeficiency virus from human T cell cultures by synergistic action of CD4-*Pseudomonas* exotoxin and reverse transcriptase inhibitors. *Proc. Natl. Acad. Sci. USA* **87**, 8889-8893.

ATTAYA, M., JAMESON, S., MARTINEZ, C.K., HERMEL, E., ALDRICH, C., FORMAN, J., FISHER LINDAHL, K., BEVAN, M.J., AND MONACO, J.J. (1992). *Ham-2* corrects the class I antigen processing defect in RMA-S cells. *Nature* **355**, 647-649.

BAKKE, O., AND DOBBERSTEIN, B. (1990). MHC class II-associated invariant chain contains a sorting signal for endosomal compartment.*Cell* **63**, 707-716.

BALZARINI, J., NAESENS, L., HERDEWIJN, P., ROSENBERG, I., HOLY, A., PAUWELS, R., BABA, M., JOHNS, D.G., AND DECELRQ, E. (1989). Marked *in vivo* antiretrovirus activity of 9-(2-phophonylmethoxy-ethyl)adenine, a selective anti-human immunodeficiency virus agent. *Proc. Natl. Acad. Sci. USA* **86**, 332-336.

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

BENNETT, K., LEVINE, T., ELLIS, J.S., PEANASKY, R.J., SAMLOFF, I.M., KAY, J., AND CHAIN, B.N. (1992). Antigen processing for presentation by class II major histocompatibility complex requires cleavage by cathepsin E. *Eur. J. Immunol.* **22**, 1519-1524.

BENNINK. J.R., AND YEWDELL, J.E. (1988). Murine cytotoxic T lymphocyte recognition of individual influenza virus proteins. High frequency of non-responder MHC class I alleles. *J. Exp. Med.* **168**, 1935-1939.

BENOIST, C., AND MATHIS, D. (1989). Positive selection of the T cell repertoir - where and when does it occur. *Cell* **58**, 1027-1033.

BENVENISTE, R.E., MORTON, W.R., CLARK, E.A., TSAI, C.-C., OCHS, H.D., WARD, J.M., KULLER, L., KNOTT, W.B., HILL, R.W., GALE, M.J., AND THOULESS, M.E. (1988). Inoculation of baboons and macaques with simian immunodeficiency virus/Mne, a primate lentivirus closely related to human immunodeficiency virus type 2. *J. Virol.* **62**, 2091-2101.

BERNARDS, R., SCHRIER, P.I., BOS, J.L., AND VAN DER EB, A.J. (1983). Role of adenovirus type 5 and type 12 early region 1B tumor antigens in oncogenic transformation. *Virology* **127**, 45-53.

BITTLE, J.L., HOUGHTEN, R.A., ALEXANDER, H., SHINNICK, T.M., SUTCLIFFE, J.G., LERNER, R.A., ROWLANDS, D.J., AND BROWN, F. (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* **298**, 30-33.

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

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

BLUESTONE, J.A., CRON, R.Q., BARRETT, T.A., HOULDEN, B., SPERLING, A.I., DENT, A., HEDRICK, S., RELLAHAN, B.M., AND MATIS, L.A. (1991). Repertoire development and ligand specificity of murine TCR gamma-delta cells. *Immunol. Rev.* **120**, 5-33.

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

BODMER, H.C., GOTCH, F.M., AND MCMICHAEL, A.J. (1989). Class I-cross-restricted T cells reveal low responder allele due to processing of viral antigen. *Nature* **337**, 653-655.

BOLOGNESI, D.P. (1990). Fresh pathways to follow. *Nature* **344**, 818-819.

BOOG, C.J., NEEFJES, J.J., BOES, J., PLOEGH, H.J., AND MELIEF, C.J. (1989). Specific immune responses restored by alteration in carbohydrate chains of surface molecules an antigen-presenting cells. *Eur. J. Immunol.* **19**, 537-542.

BOON, T., AND VAN PEL, A. (1989). T cell-recognized antigenic peptides derived from the cellular genome are not protein degradation products but can be generated directly by transcription and translation of short subgenomic regions. *Immunogenetics* **29**, 75-79.

BOULOT, G., EISELE, J.-L., BENTLEY, G.A., BHAT, T.N., WARD, E.S., WINTER, G., AND POLJAK, R.J. (1990). Crystallization and preliminary X-ray diffraction study of the bacterially expressed Fv from the monoclonal anti-lysozyme antibody D1.3 and of its complex with antigen, lysozyme. *J. Mol. Biol.* **213**, 617-619.

BROOKS, A., HARTLY, S., KJER-NIELSEN, L., PERERA, J., GOODNOW, C.C., BASTEN, A., AND MCCLUSKEY, J. (1991). Class II-restricted presentation of an endogenously derived immunodominant T cell determinant of hen egg lysozyme. *Proc. Natl. Acad. Sci. USA* **88**, 8.

BROWN, M.G., DRISCOLL, J., AND MONACO, J.J. (1991). Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complexes. *Nature* **353**, 355-357.

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 histocompatibilty antigen HLA-DR1. *Nature* **364**, 33-39.

BROWNE, H., SMITH, G., BECH, S., AND MINSONT, A. (1990). A complex between the MHC class I homologue encoded by human cytomegalovirus and beta 2 microglobulin. *Nature* **347**, 770-772.

BRUNT, L.M., PORTNOY, D.A., AND UNANUE, E.R. (1990). Presentation of *Listeria monocytogenes* to $CD8^+$ T cells requires secretion of hemolysin and intracellular bacterial growth. *J. Immunol.* **145**, 3540-3546.

BUBBERS, J.R., AND LILLY, F. (1977). Selective incorporation of H-2 antigenic determinants into Friend virus particles. *Nature* **266**, 458-459.

BURGERT, H.-G., MARYANSKI, J.L., AND KVIST, S. (1987). "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc. Natl. Acad. Sci. USA* **84**, 1356-1360.

CAPON, D.J., CHAMOW, S.M., MORDENTI, J., MARSTERS, S.A., GREGORY, T., MITSUYA, H., BYRN, R.A., LUCAS, C., WURM, F.M., GROOPMAN, J.E., BRODER, S., AND SMITH, D.H. (1989). Designing CD4 immunoadhesins for AIDS therapy. *Nature* **337**, 525-531.

CARBONE, F., MOORE, M., SHEIL, J., AND KNIGHT, S.C. (1988). Induction of cytotoxic T lymphocytes by primary *in vitro* stimulation with peptides. *J. Exp. Med.* **167**, 1767-1780.

CAZENAVE, P.-A., MARCHE, P.N., JOUVIN-MARCHE, E., VOEGTLE, D., BONHOMME, F., BANDEIRA, A., AND COUTINHO, A. (1990). $V\beta17$ gene polymophism in wild-derived mouse strains: two amino acid substitutions in the $V\beta17$ region greatly alter T cell receptor specificity. *Cell* **63**, 717-728.

CERUNDOLO, V., ALEXANDER, J., ANDERSON, K., LAMB, C., CRESSWELL, P., MCMICHAEL, A., GOTH, F., AND TOWNSEND, A. (1990). Presentation of viral antigen controlled by a gene in the major histocompatibility complex. *Nature* **245**, 449-452.

CHAN, W.L., RODGERS, A., HANCOCK, R., TAFFS, F., KITCHIN, P., FARRAR, G., AND LIEW, F.Y. (1992). Protection in SIV-vaccinated monkeys correlates with anti-HLA class I antibody response. *J. Exp. Med.* **176**, 1203-1207.

CHEN, B.P., AND PARHAM, P. (1989). Direct binding of influenza peptides to class I HLA molecules. *Nature* **337**, 743-745.

CHICZ, R.M., URBAN, R.G., LANE, W.S., GORGA, J.C., STERN, L.J., VIGNALI, D.A.A., AND STROMINGER, J.L. (1992). Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogenous in size. *Nature* **358**, 764-768.

CHOI, Y., KAPPLER, J.W., AND MARRACK, P. (1991). A superantigen encoded in the open reading frame of the 3' long terminal repeat of mouse mammary tumour virus. *Nature* **350**, 203-207.

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.

CHRISTINK, E.R., LUSCHER, M.A., BARBER, B.H., AND WILLIAMS, D.B. (1991). Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature* **352**, 67-70.

CLAPHAM, P.R., WEBER, J.N., WHITBY, D., MCINTOSH, K., DALGLEISH, A.G., MADDON, P.J., DEEN, K.C., SWEET, R.W., AND WEISS, R.A. (1989). Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T-cells and monocytes but not for brain and muscle cells. *Nature* **337**, 368-370.

CLAVEL, F., MANSINHO, K., CHAMARET, S., GUETARD, D., FAVIER, V., NINA, J., SANTOS-FERREIRA, M.O., CHAMPALIMAUD, J.L., AND MONTAGNIER, L. (1987). Human immunodeficiency virus type 2 infection associated with AIDS in West Africa. *N. Engl. J. Med.* **316**, 1180-1185.

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.

COATES, J.A.V., CAMMACH, N., JENKINSON, H.J., JOWETT, A.J., JOWETT, M.I., PEARSON, B.A., PENN, C.R., ROUSE, P.L., VINER, K.C., AND CAMERON, J.M. (1992). '-deoxy-3'-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. *Antimicrob. Agents Chemother.* **36**, 733-739.

COHEN, G.H., DIETZSCHOLD, B., PONCE DE LEON, M., LONG, D., GOLUB, E., VARRICHIO, A., PEREIRA, L., AND EISENBERG, R.J. (1984). Localization and synthesis of an antigenic deteminant of herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibody. *J. Virol.* **49**, 102-108.

COLEBUNDERS, R., *et al.* (1992). Proceedings from *The 8th International Conference on AIDS*, Amsterdam, p. B 89.

COLLINS, D., UNANUE, E.R., AND HARDING, C.V. (1991). Reduction of disulfide bonds in lysosomes is a key step in antigen processing. *J. Immunol.* **147**, 4054-4059.

COUPAR, B.E.H., ANDREW, M.E., BOTH, G.W., AND BOYLE, D.B. (1986). Temporal Regulation of influenza haemagglutinin expression in vaccinia virus recombinants and effects on the immune response. *Eur. J. Immunol.* **16**, 1479-1487.

CRANAGE, M.P., ASHWORTH, L.A.E., GREENAWAY, P.J., MURPHY-CORB, M., AND DESROSIERS, R. (1992) AIDS vaccine development. *Nature* **355**, 685-686.

CRANAGE, M.P., POLYANSKAYA, N., MCBRIDE, B., COOK, N., ASHWORTH, L.A.E., DENNIS, M., BASKERVILLE, A., GREENAWAY, P.J., CORCORAN, T., KITCHIN, P., ROSE, J., MURPHEY-CORB, M., DESROSIERS, R.C., STOTT, E.J., AND FARRAR, G.H. (1993). Studies on the specificity of the vaccine effect elicited by inactivated simian immunodeficiency virus. *AIDS Res. Hum. Retro.* **9**, 13-22.

DAAR, E.S., LI, X.L., MOUDGIL, T., AND HO, D.D. (1990). High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. *Proc. Natl. Acad. Sci. USA* **87**, 6574-6578.

DALGLEISH, A.G., BEVERLEYM, P.C., CLAPHAM, P.R., CRAWFORD, D.H., GREAVES, M.F., AND WEISS, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**, 763-767.

DANIEL, M.D., KIRCHOFF, F., CZAJAK, S.C., SEHGAL, P.K., AND DESROSIERS, R.D. (1992). Protective efforts of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**, 1938-1941.

DAVIS, M.M., AND BJORKMAN, P.J. (1988). T cell antigen receptor genes and T cell recognition *Nature* **334**, 395-401.

DEGEN, E., AND WILLIAMS, D.B. (1991). Participation of a novel 88 kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell. Biol.* **112**, 1099-1115.

DELAMARTER, J.F., MERMOD, J.J., LIANG, C.M., ELIASON, J.F., AND THATCHER, D.R. (1985). Recombinant murine GM-CSF from *E. coli* has biological activity and is neutralized by a specific antiserum. *EMBO J.* **4**, 2575-2581.

DELISI, C., AND BERZOFSKY, J.A. (1985). T cell antigenic sites tend to be amphipathic structures. *Proc. Natl. Acad. Sci. USA* **82**, 7048-7052.

DELLABONA, P., PECCOUD, J., KAPPLER, J., MARRACK, P., BENOIST, C., AND MATHIS, D. (1990). Superantigens interact with MHC class II molecules outside of the antigen groove. *Cell* **62**, 1115-1121.

DERES, K., SCHILD, H., WIESMULLER, K.H., JUNG, G., AND RAMMENSEE, H.G. (1989). *In vivo* priming of virus-specific cyto-toxic lymphocytes-T with synthetic lipopeptide vaccine. *Nature* **342**, 561-564.

DESROSIERS, R.C., WYAND, M.S., KODAMA, 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.

DEUWEKE, T.J., *et al.* (1993). *Proc. Natl. Acad. Sci. USA* In press.

DEVERSON, E.V., COADWELL, W.J., MONACO, J.J., BUTCHER, G.W., AND HOWARD, J.C. (1990). MHC class I region encoding proteins related to the multidrug resistance family of transmembrane transporters. *Nature* **248**, 738-741.

DONAHUE, P.R., KORNFELD, H., GALLO, R.C., AND MULLINS, J. (1988). Unpublished. (Sequence available through the GenBank database.)

DYSON, P.J., KNIGHT, A.M., FAIRCHILD, S., SIMPSON, E., AND TOMONARI, K. (1991). Genes encoding ligands for deletion of V beta 11 T cells co-segregate with mammary tumour virus genomes. *Nature* **349**, 531-532.

ELLOUZ, F., ADAM, A., CIOBARU, R., AND LEDERER, E. (1974). Minimal structure requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys. Res. Commun.* **59**, 1317-1321.

EMBRETSON, J., ZUPANCIC, M., RIBAS, J.L., BURKE, A., RACZ, P., TENNER-RACZ, K., AND HAASE, A.T. (1993). Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362**, 359-362.

EMINI, E.A., JAMESON, B.A., AND WIMMER, E. (1983). Priming for and induction of anti-polio neutralizing antibodies by synthetic peptides. *Nature* **304**, 699-703.

ERICKSON, J., NEIDHART, D.J., VANDRIE, J., KEMPF, D.J., WANG, X.C., NORBECK, D.W., PLATTNER, J.J., RITTENHOUSE, J.W., TURON, M., WIDEBURG, N., KOHLBRENNER, W.E., SIMMER, R., HELFRICH, R., PAUL, D.A., AND KNIGGE, M. (1990). Design, activity, and 2.8 Å crystal structure of a C2 symmetrical inhibitor complexed to HIV-1 protease. *Science* **249**, 527-533.

FALK, K., ROTZSCHKE, O., AND RAMMENSEE, H.-G. (1990). Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature* **348**, 248-251.

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.

FARNET, C., AND HASELTINE, W. (1990). Integration of human immunodeficiency virus type 1 DNA in vitro. *Proc. Natl. Acad. Sci. USA* **87**, 4164-4168.

FAZELY, F., DEZUBE, B.J., ALLEN-RYAN, J., PARDEE, A.B., AND RUPRECHT, R.M. (1991). Pentoxyfylline (Trental) decreases the replication of the human immunodeficiency virus type 1 in human peripheral blood mononuclear cells and in cultured T cells. *Blood* **77**, 1653-1656.

FERNS, R.B., TEDDER, R.S. AND WEISS, R.A. (1987). Characterization of monoclonal antibodies against the human immunodeficiency virus (HIV) *gag* products and their use in monitoring HIV isolate variation. *J. gen. Virol.* **68**, 1543-1551.

FESTENSTEIN, H. (1973). Immunogenic and biological aspects if *in vitro* lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* **15**, 29-56.

FETTEN, J.V., ROY, N., AND GILBOA, E. (1991). A frameshift at the NH_2-terminus of the nucleoprotein gene does not affect generation of cytotoxic T lymphocyte epitopes. *J. Immunol.* **147**, 2697-2705.

FLAVELL, R.A., ALLEN, H., BURKLY, L.C., SHERMAN, D.H., WANECK, G.L., AND WIDERA, G. (1986). Molecular biology of the H-2 histocompatibility complex. *Science* **233**, 437-443.

FLEISCHER, B., AND SCHREZENMEIER, H. (1988). T cell stimulation by staphylococcal enterotoxins. *J. Exp. Med.* **167**, 1697-1707.

FLEISCHER, B., GERARDY, R., METZROTH, B., CARREL, S., GERLACH, D., AND KOHLER, W. (1991). an evolutionary conserved mechanism of T cell activation by microbial toxins. *J. Immunol.* **146**, 11-17.

FRANKEL, W.N., RUDY, C., COFFIN, J.M., AND HUBER, B.T. (1991). Linkage of MLS genes to endogenous mammary tumour viruses of inbred mice. *Nature* **249**, 526-528.

FRASER, J.D. (1989). High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. *Nature* **339**, 221-224.

FREMONT, D.H., MATSAMURA, M., STURA, E.A., PETERSON, P.A., AND WOLSON, I.A. (1992). Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science* **257**, 91-927.

FREUDENTHAL, P.S., AND STEINMAN, R.M. (1990). The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. USA* **87**, 7698-7702.

FUKASAWA, M., MIURA, T., HASEGAWA, A., MORIKAWA, S., TSUJIMOJO, H., KITAMURA, T., AND HAYAMI, M. (1988). Sequence of simian immunodeficiency virus from Afircan green monkey, a new member of the HIV-SIV group. *Nature* **333**, 457-461.

FULEIHAN, R., MOURAD, W., GEHA, R.S., AND CHATILA, T. (1991). Engagement of MHC class II molecules by staphylococcal exotoxins delivers a co-mitogenic signal to human B cells. *J. Immunol.* **146**, 1661-1666.

FULTZ, P.N., MCCLURE, H.M., ANDERSON, D.C., SWENSON, R.B., ANAND, R., AND SRIVASAN, A. (1986). Isolation of a T-lymphotropic retrovirus from naturally infected soothy mangabey monkeys (*Cercocebus atys*). *Proc. Natl. Acad. Sci. USA* **83**, 5286-5290.

GAMMON, G., SHASTRI, N., COGSWELL, J., WILBUR, S., SADEGHNASSERI, S., KRZYCH, U., MILLER, A., AND SERCARZ, E. (1987). The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Rev.* **98**, 53-75.

GAO, L., WAGNER, E., COTTEN, M., AGARWAL, S., HARRIS, C., RØMER, M., MILLER, L., HU, P.-C., AND CURIEL, D. (1993a). Direct *in vivo* gene transfer to airway epithelium employing adenovirus-polylysine-DNA complexes. *Hum. Gene Ther.* **4**, 17-24.

GAO, W.-Y., SHIRASAKA, T., JOHNS, D.G., BRODER, S., AND MITSUYA, H. (1993a). Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral-blood mononuclear cells. *J. Clin. Invest.* **91**, 2326-2333.

GARDNER, M.B., AND MURPHY-CORB, M. (1990). Vaccine protection of rhesus monkeys against homologous and heterologous strains of SIV. Proceedings from *6h Cent Gardens Meeting, Marnes-la Coquette, France*. pp. 221-226.

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.

GASCOIGNE, N.R., AND AMES, K.T. (1991). Direct binding of secreted T cell receptor beta chain to superantigen associated with class II major histocompatibility complex protein. *Proc. Natl. Acad. Sci. USA* **88**, 613-616.

GASKINS, H.R., MONACO, J.J., AND LEITER, E.H. (1992). Expression of intra-MHC transporter (*HAM*) genes and class I antigens in diabetes-susceptible NOD mice. *Science* **256**, 1826-1828.

GELDERBLOM, H., REUPKE, H., WINKEL, T., KUNZE, R., AND PAULI, G. (1987). MHC antigens: constituents of the envelopes of human and simian immunodeficiency viruses. *Z Naturforsch* {C} **42**, 1328-1334.

GERMAIN, R.N., AND HENDRIX, L.R. (1991). MHC class II structure, occupancy and surface expression determined by port-endoplasmic reticulum antigen binding. *Nature* **353**, 134-139.

GIAVEDONI, L.P., PLANELLES, V., HAIGWOOD, N.L., AHMED, S., KLUGE, J.D., MARTHA, M.L., GARDNENR, M.B., LUCIW, P.A., AND YILMA, T.D. (1993). Immune response of rhesus macaques to recombinant simian immunodeficiency virus gp130 does not protect from challenge infection. *J. Virol.* **67**, 577-583.

GINSBERG. H.S., LUNDHOLM-BEAUCHAMP, U., HORSTWOOD, R.L., PERNIS, B., WOLD, W.S.M., CHANOCK, R.M., AND PRINCE, G.A. (1989). Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA* **86**, 3823-3827.

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.

GOTTESMANN, S., HALPERN, E., AND TRISLER, P. (1981). Role of *sulA* and *sulB* in filamentation by *lon* mutants of *Escherichia coli* K-12. *J. Bacteriol.* **148**, 265-273.

GOTTLIEB, M.S., SCHROFF, R., SCHANKER, H.M., WEISMAN, J.D., FAN, P.T., WOLF, R.A., AND SAXON, A. (1981). *Pneumocistis carnii* pneumonia and mucosal candidis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N. Engl. J. Med.* **305**, 1425-1431.

GRITZ, L., AND DAVIES, J. (1983). Plasmid-encoded hygromycin B resistance: The sequence of hygromycin B-phosphotransferase gene and its expression in *E. coli* and *S. cerevisiae*. *Gene* **25**, 179-188.

GUAGLIARDI, L.E., KOPPELMAN, B., MARKS, M.S., CRESSWELL, P., AND BRODSKY, F.M. (1990). Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. *Nature* **343**, 133-139.

GUO, H.-G., JARDETZKY, T.S., GARRETT, T.P.J., LANE, W.S., STROMINGER, J.L., AND WILEY, D.C. (1992). Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature* **360**, 364-366.

GUPTA, R.K., RELYVELD, E.H., LINDBLAD, E.B., BIZZINI, B., BEN-EFRAIM, S., AND GUPTA, C.K. (1993). Adjuvants - a balance between toxicity and adjuvanticity. *Vaccine* **11**, 293-306.

HABESHAW, J., HOUNSELL, E., AND DALGLEISH, A. (1992). Does the HIV envelope induce a chronic graft-versus-host-like disease? *Immunol. Today* **13**, 207-210.

HALLENBERGER, S., BOSCH, V., ANGLIKER, H., SHAW, E., KLENK, H.D., AND GARTEN, W. (1992). Inhibition of furine-mediated cleavage activation of HIV gp160. *Nature* **360**, 358-361.

HANKE, T., GRAHAM, F.L., LULITANOND, V., AND JOHNSON, D. (1990). Herpes simplex IgG Fc receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. *Virology* **177**, 437-444.

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., COLLINS, D.S., SLOT, J.W., GEUZE, H.J., AND UNANUE, E.R. (1991a). Liposome-encapsulated antigens are processed in lysosomes, recycled and presented to T cells. *Cell* **64**, 393-401.

HARDING, C.V., COLLINS, D.S., KANAGAWA, O., AND UNANUE, E.R. (1991b). Liposome-encapsulated antigen engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation. *J. Immunol.* **147**, 2860-2863.

HASELTINE, W.A. (1991). Molecular biology of the human immunodeficiency virus type 1. *FASEB J.* **5**, 2349-2360.

HASSELOFF, J., AND GERLACH, W.L. (1988). Simple RNA enzymes with new and highly specific endoribonuclease activity. *Nature* **334**, 585-591.

HAYNES, B.F., ARTHUR, L.O., FROST, P., MATTHEWS, T.J., LANGLOIS, A.J., PALKER, T.J., HART, M.K., SCEARCE, R.M., JONES, D.M., MCDANAL, C., OTTINGER, J., BOLOGNESI, D.P., AND WIENHOLD, K.J. (1993). Conversion of an immunogenic human immunodeficiency virus (HIV) envelope synthetic peptide to a tolerogen in chimpanzees by the fusogenic domain of HIV gp41 envelope protein. *J. Exp. Med.* **177**, 717-727.

HEBER-KATZ, E., AND ACHA-ORBEA, H. (1989). The V-region disease hypothesis: evidence from autoimmune encephalomyelitis. *Immunol. Today* **10**, 164-169.

HELBERT, M.R., L'AGE-STEHR, J., AND MITCHISON, N.A. (1993). Antigen presentation, loss of immunological memory and AIDS. *Immunol. Today* **14**, 340-344.

HENDERSON, R.A., MICHEL, H., SAKAGUCHI, K., SHABANOWITZ, J., APPELLA, E., HUNT, D.F., ENGELHARD, V.H. (1992). HLA-A2.1-associated peptides from a mutant cell line - a second pathway of antigen presentation. *Science* **255**, 1264-1266.

HEUCKEROTH, R.O., JACKSON-MACHELSKI, E., ADAMS, S.P., KISHORE, N.S., HUHN, M., KATOH, A., LU, T., GOKEL, G.W., AND GORDON, J.I. (1990). Novel fatty acyl substrates for myristoyl-CoA-protein *N*-myristoyl-transferase *J. Lipid Res.* **31**, 1121-1129.

HO, D.D., ROTA, T.R., KAPLAN, J.C., HARTSHORN, K.L., ANDREWS, C.A., SCHOOLEY, R.T., AND HIRSCH, M.S. (1985). Recombinant human interferon alfa-A suppresses HTLV-III replication *in vitro*. *Lancet* **1**, 602-604.

HOCHSTENBACH, F., DAVID, V., WATKINS, C., AND BRENNER, M.B. (1992). Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T and B cell antigen receptors and major histocompatibilty complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA* **89**, 4734-4738.

HOSKEN, N.A., AND BEVAN, M.J. (1990). Defective presentation of endogenous antigen by a cell line expressing class I molecules. *Science* **340**, 443-448.

HOVANESSIAN, A.G., KRUST, B., JACOTOT, E., AND CALLEBAUT, C. (1993). Entry of HIV into CD4+ cells requires the T-cell activation antigen CD26. Proceedings from *The 8th Cent Gardens Meeting, Marnes-la Coquette, France*. p. 80.

HSU, M.-C., DHINGRA, U., EARLY, J.V., HOOLY, M., KEITH, D., NALIN, C.M., RICHOU, A.R., SCHUTT, A.D., TAM, S.Y., POTASH, M.J., VOLSKY, D.J., AND RICHMAN, D.D. (1993) Inhibition of type 1 human immunodeficiency virus replication by a tat antagonist to which the virus remains sensitive after prolonged exposure *in vitro*. *Proc. Natl. Acad. Sci. USA* **90**, 6395-6399.

HU, S.-L., ABRAMS, K., BARBER, G.N., MORAN, P., ZARLING, J.M., LANGLOIS, A.J., KULLER, L.R., MORTON, W.R., AND BENVENISTE, R.E. (1992). Protection of macaques against SIV infection by subunit vaccines of SIV envelope glycoprotein gp160. *Science* **255**, 456-459.

HUGIN, A.W., VACCHIO, M.S., AND MORSE, H.C. (1991). A virus-encoded 'superantigen' in a retrovirus-induced immunodeficiency syndrome of mice. *Science* **252**, 424-427.

HUNT, D.F., AND ENGELHARD, V.H. (1992). HLA-A2.1-associated peptides from a mutant cell line - a 2nd pathway of antigen presentation. *Science* **255,** 1264-1266.

HUNT, D.F., HENDERSON, R.A., SHABANOWITZ, J., SAKAGUCHI, K., MICHEL, H., SEVILIR, N., COX, A.L., APPELLA, E. AND ENGELHARD, V.H. (1992). Characterization of peptides bound to the class I MHC molecules HLA-A2.1 by mass spectrometry. *Science* **255**, 1261-1263.

HYDE, S.C., GILL, D.R., HIGGINS, C.F., TREZISE, A.E.O., MACVINISH, L.J., CUTHBERT, A.W., RATCLIFF, R., EVANS, M.J., AND COLLEDGE, W.H. (1993). Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature* **362**, 250-255.

ISAACS, J.D., WATTS, R.A., HAZLEMAN, B.L., HALE, G., KEOGAN, M.T., COBBOLD, S.P., AND WALDMANN, H. (1992). Humanized monoclonal antibody therapy for rheumatoid arthritis. *Lancet* **26,** 748-752.

IZANT, J.G., AND WEINTRAUB, H. (1985). Constitutive and conditional suppression of exogenous and endogenous genes by antisense RNA. *Science* **229**, 345-352.

JACKS, T., POWER, M.D., RASIARZ, F.R. LUCIW, P.A. BARR, P.J., AND VARMUS, H. (1988). Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression. *Nature* **231**, 280-283.

JACOBSEN, S., SEKALY, R.P., JACOBSEN, C.L., MCFARLAND, H.F., AND LOY, E.D. (1989). HLA class II-restricted presentation of cytoplasmic measles virus antigen to cytotoxic T cells. *J. Virol.* **63**, 1756-1763.

JANEWAY, C.A. JR., CONRAD, P.J., TITE, J., JONES, B., AND MURPHY, D.B. (1983). Efficiency of antigen presentation differs in mice differing at the *Mls* locus. *Nature* **306**, 80-82.

JANEWAY, C.A., YAGI, J., JR., CONRAD, P.J., KATZ, M.E., JONES, B., VROEGOP, S., AND BRUXSER, S. (1989). T cell responses to *Mls* and to bacterial proteins that mimic its behaviour. *Immunol. Rev.* **107**, 61-65.

JARDETZKY, T.S., GORGA, J.C., BUSH, R., ROTHBARD, J., STROMINGER, J.L., AND WILEY, D.C. (1990). Peptide binding to HLA-DR1: a peptide with most residues substituted to alanine retains MHC binding. *EMBO J.* **9**, 1797-1803.

JARDETZKY, T.S., LANE, W.S., ROBINSON, R.A., MADEN, D.R., AND WILEY, D.C. (1991). Identification of self peptides bound to purified HLA-B27. *Nature* **353**, 3135-3139.

JEFFERIES, W.A., AND BURGERT, H.G. (1990). E3/19K from adenovirus 2 is an immuno-subversive protein that binds to a structural motif regulating the intracellular transport of major histocompatibility complex class I proteins. *J. Exp. Med.* **172**, 1653-1664.

JENSEN, P.E. (1990). Regulation of antigen presentation by acidic pH. *J. Exp. Med.* **171**, 1779-1784.

JENSEN, P.E. (1991). Reduction of disulfide bonds during antigen processing: evidence from a thiol-dependent insulin determinant. *J. Exp. Med.* **174**, 1121-1130.

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 antigens. *J. Exp. Med.* **168**, 293-296.

JOHNSON, P.R., MONTEFIORI, D.C., GOLDSTEIN, S., HAMM, T.E., ZHOU, J., KITOV, S., HAIGWOOD, N.L., MISHER-LONDON, W.T., GERIN, J.L., ALLISON, A., PURCELL, R.H., CHANOCK, R.M., AND HIRSCH, V.M. (1992a). Inactivated whole-virus vaccine derived from a proviral DNA clone of simian immunodeficiency virus induces high levels of neutralizing antibodies and confers protection against heterologous challenge. *Proc. Natl. Acad. Sci. USA* **89**, 2175-2179.

JOHNSON, P.R., MONTEFIORI, D.C., GOLDSTEIN, S., HAMM, T.E., ZHOU, J., KITOV, S., HAIGWOOD, N.L., MISHER-LONDON, W.T., GERIN, J.L., ALLISON, A., PURCELL, R.H., CHANOCK, R.M., AND HIRSCH, V.M. (1992b). Inactivated whole-virus vaccine in macaques: Evaluation of protective efficacy against challenge with cell-free virus infected cells. *AIDS Res. Hum. Retro.* **8**, 1501-1505.

JONES, S.T., AND BENDIG, M.M. (1991). Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. *Biotechnology* **9**, 88-89.

JORGENSEN, J.L., ESSER, U., DESTGROTH, B.F., REAY, P.A., AND DAVIS, M.M. (1992). Mapping T cell receptor peptide contacts by variant peptide immunization of single-chain transgenics. *Nature* **355**, 224-230.

KABAT, E.A., WU, T.T., REID-MILLER, M., PERRY, H.M., AND GOTTESMANN, K.S. (1987). Sequences of proteins of immunological interest. U.S. Department of Health and Human Services, U.S. Government Printing Offices.

KAO, F.-T., AND PUCK, T.T. (1968). Genetics of somatic mammalian cells. VII. Induction and isolation of nutritional mutants in Chinese hamster cells. *Proc. Natl. Acad. Sci. USA* **60**, 1275-1281.

KESSLER, S.W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**, 1617-1624.

KIM, H.W., KEIKIN, S.L., AND ARROBIO, J.O. (1976). Cell-mediated immunity to respiratory syncytial virus induced by inactivated vaccine or by infection. *Pediatric Res.* **10**, 74-78.

KITTS, P.A., AYRES, M.D., AND POSSEE, R.D. (1990). Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res.* **18**, 5667-5672.

KLAUS, G.G.B., AND HUMPHREY, J.H. (1977). The generation of memory cells. *Immunology* **33**, 31.

KORNFELD, H., RIEDEL, N., VIGLIANTI, G.A., HIRSCH, V., AND MULLINS, J.I. (1987). Cloning of HTLV-4 and its relation to simian and human immunodeficiency viruses. *Nature* **326**, 610-613.

KOTWAL, G.J., AND MOSS, B. (1989). Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. *J. Virol.* **63**, 600-606.

KOUTTAB, N., ACCETTA, G., CALABRESI, P., AND SKOWRON, G. (1992). Proceedings from *The 8th International Conference on AIDS*, Amsterdam, p. B 161.

KRIEGER, J.I., KARR, R.W., GREY, H.M., YU, W.Y., O'SULLIVAN, D., BATOVSKY, L., ZHENG, Z.L., COLON, S.M., GAETA, F.C.A., AND SIDNEY, J. (1991). Single amino acid changes in DR and antigen define residues critical for peptide-MHC binding and T cell recognition. *J. Immunol.* **146**, 2331-2340.

KROPSHOFER, R., MAX, H., MULLER, C.A., HESSE, F., STEVANOVIC, S., JUNG, G., AND KALBACHER, H. (1992). Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. *J. Exp. Med.* **175**, 1799-1803.

KUNKEL, T.A., ROBERTS, J.D., AND ZAKOUR, A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Method Enzymol.* **154**, 367-382.

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.

KVIST, S., OSTBERG, L., PERSSON, H., PHILIPSON, L., AND PETERSON, P.A. (1978). Molecular association between transplantation antigens and cell surface antigen in adenovirus-transformed cell line. *Proc. Natl. Acad. Sci. USA* **75**, 5674-5678.

LANGLOIS, A.J., WEINHOLD, K.J., MATTHEWS, T.H., GREENBEGR, M.L., AND BOLOGNESI, D.P. (1992). Detection of antihuman cell antibodies in sera from macaques immunized with whole inactivated virus. *AIDS Res. Hum. Retro.* **8**, 1641-1652.

LE GRAND, R., VOGT, G., VASLIN, B., ROQUES, P., THEODORO, F., AUBERTIN, A.-M., AND DORMONT, D. (1992). Specific and non-specific immunity and protection of macaques against SIV infection. *Vaccine* **10**, 873-879.

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 characterization of potential glycosylation sites of the type 1 recombinant human-immunodeficiency-virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **265**, 10373-10382.

LETVIN, N.L., DANIEL, M.D., SEHGAL, P.K., DESROSIERS, R.C., HUNT, R.D., WALDRON, L.M., MACKEY, J.J., SCHMIDT, D.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.

LEVER, A., GOTTLINGER, H., HASELTINE, W.A., AND SODROSKI, J. (1989). Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J. Virol.* **63**, 4085-4087.

LEVY, J.A. (1993). Pathogenesis of human human immunodeficiency virus infection. *Micribiol. Rev.* **57**, 183-289.

LEVY, F., GABATHULER, R., LARSSON, R., AND KVIST, S. (1991). ATP is required for *in vitro* assembly of MHC class I antigens but not for transfer of peptides across the ER membrane. *Cell* **67**, 265-274.

LEWIS, M.G., ELKINS, W.R., MCCUTCHAN, E.F., BENVENISTE, R.E., LAI, C.Y., MONTEFIORI, D.C., BURKE, D.S., EDDY, G.A., AND SCHAFFERMAN, A. (1993). Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. *Vaccine* **11**, 1347-1355.

LI, J., LORD, C.I., HASELTINE, W., LETVIN, N.L., AND SODROSKI, J. (1992). Infection of cynomolgus macaques with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoprotein. *J. AIDS* **5**, 639-646.

LIFSON, J.D., FEINBERG, M.R., REYES, G.R., RABIN, L., BASIRIPOUR, B., CHAKRABARTI, S., MOSS, B., WONG-STAAL, F., STEIMER, K.S., AND ENGLEMAN, E.G. (1986). Induction of CD4-dependent cell fusion by HTLV-III/LAV envelope glycoprotein. *Nature* **323**, 725-728.

LINSLEY, P.S., BRADY, W., GROSMAIRE, L, ARUFFO, A., DAMLE, N.K., AND LEDBETTER, J.A. (1991). Binding of the B cell activation antigen B7 to CD28 co-stimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* **173**, 721-730.

LJUNGGREN, H.G., STAM, N.J., OHLEN, C., NEEFJES, J.J., HOGLUND, P., HEEMELS, M.T., BASTIN, J., SCHUMACHER, T.N.M., TOWNSEND, A., KARRE, K., AND PLOEGH, H.L. (1990). Empty MHC class I molecules come out in the cold. *Nature* **346**, 476-480.

LOTTEAU, V., TEYTON, L., PELERAUX, A., NILSSON, T., KARLSSON, L., SCHMID, S.L., QUARANTA, V., AND PETERSON, P.A. (1991). Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600-605.

LOVELAND, B., WANG, C.R., YONEKAWA, H., HERMEL, E. AND LINDAHL, K.F. (1990). Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. *Cell* **60**, 971-980.

MACATONIA, S.E., TAYLOR, P.M., KNIGHT, S.C., AND ASKONAS, B.A. (1989). Primary stimulation by dendritic cells induces anti-viral proliferative and cytotoxic T cell responses *in vitro*. *J. Exp. Med.* **169**, 1255-1264.

MACATONIA, S.E., PATTERSON, S., AND KNIGHT, S.C. (1991). Primary proliferative and cytotoxic T cell responses to HIV induced *in vitro* by human dendritic cells. *Immunology* **74**, 399-406.

MADDEN, D.R., GORGA, J.C., STROMINGER, J.L., AND WILEY, D.C. (1991). The structure of HLA-27 reveals nonamer self-peptides bound in an extended conformation. *Nature* **353**, 321-325.

MANCA, F., HABESHAW, J.A., AND DALGLEISH, A.G. (1990). HIV envelope glycoprotein, antigen specific T-cell responses, and soluble CD4. *Lancet* **335**, 811-815.

MANIATIS, T., FRITSCH, E.F., AND SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. New York, Cold Spring Harbor Laboratory.

MARIE, C., AND SPAHR, P.I. (1986). Rouse sarcoma virus nucleic acid-binding protein p12 is necessary for viral 70S RNA dimer formation and packaging. *J. Virol.* **60**, 450-459.

MARRACK, P., AND KAPPLER, J. (1990). The staphylococcal enterotoxins and their relatives. *Science* **248**, 705-711.

MARRACK, P., KUSHNIR, E., AND KAPPLER, J. (1991). A maternally inherited superantigen encoded by a mammary tumour virus. *Nature* **249**, 524-526.

MARTHAS, M.L., SUTJIPTO, S., HIGGINS, J., LOHMAN, B., TORTEN, J., LUCIOU, P.A., MARX, P.A., AND REDERSEN, N.C. (1990). Immunization with a live attenuated simian immunodeficiency virus (SIV) prevents early, but not infection in rhesus macaques challenged with pathogenic SIV. *J. Virol.* **64**, 3694-3700.

MARYANSKI, J.L., ABASTADO, J.-P., CORRADIN, G., AND CEROTTINI, J.-C. (1989). Structural features of peptides recognized by H-2K^{d}-restricted T cells. *Cold Spring Harbor Symp. Quant. Biol.* **54**, 545-550.

MARYANSKI, J.L., VERDINI, A.S., WEBER, P.C., SALEMME, F.R., AND CORRADIN, G. (1990). Competitor analogs for defined T cell antigens: peptide incorporating a putative binding motif and polyproline or polyglycine spacers. *Cell* **60**, 63-72.

MATSAMURA, M., FREMONT, D.H., PETERSON, P., AND WILSON I.A. (1992). Emerging principles for the recognition of antigens by MHC class I molecules. *Science* **257**, 927-934.

MATSUI, K., BONIFACE, J.J., REAT, P.A., SCHILD, H., FAZEKAS DE ST.GROTH, B., AND DAVIS, M.M. (1991). Low-affinity interaction of peptide-MHC complexes with T cell receptors. *Science* **254**, 1788-1791.

MATTHES, E., LEHMANN, D., SCHOLZ, D., ROSENTHAL, H.A., AND LANGEN, P. (1988). Phopshorylation, anti-HIV activity and cyto-toxicity of 3'-fluorothymidine. *Boichem. Biophys. Res. Commun.* **153**, 825-831.

MCKEATING, J.A., GRIFFITHS, P.D., AND WEISS, R.A. (1990). HIV susceptibility conferred to human fibroblasts by cytomegalovirus-induced Fc receptor. *Nature* **343**, 659-661.

MERLUZZI, V.J., HARGRAVE, K.D., LABADIA, M., GROZINGER, K., SKOOG, M., WU, J.C., SHIH, R.J., ECKNER, K., HATTOX, S., ADAMS, J., ROSENTHAL, A.S., FAANES, R., ECKNER, R.J., KOUP, R.A., AND SULLIVAN, J.L. (1990) Inhibition of HIV-1 replication by a nonnucleoside reverse transcriptase inhibitor. *Science* **250**, 1411-1413.

MEYAARD, L., OTTO, S.A., JONKER, R.R., MIJNSTER, M.J., KEET, R.P.M., AND MIEDEMA, F. (1992). Programmed death of T cells in HIV-1 infection. *Science* **257**, 217-219.

MEYAARD, L., SCHUITEMAKER, H., AND MIEDEMA, F. (1993). T-cell disfunction in HIV infection: anergy due to defective antigen-presenting cell function? *Immunol. Today* **14**, 161-164.

MEYERHANS, A., CHEYNIER, R., ALBERT, J., SETH, M., KWOK, S., SNINSKY, J., MORFELDTMANSON, L. ASJO, B., AND WAINHOBSON, S. (1989). Temporal fluctuations in HIV quasispecies *in vivo* are not reflected by sequential HIV isolations. *Cell* **58**, 901-910.

MILLS, K.H.G., NIXON, D.F., AND MCMICHAEL, A.J. (1989). T-cell strategies in AIDS vaccines: MHC-restricted T-cell responses to HIV proteins. *AIDS* **3** (suppl. 1), S101-S110.

MILLS, K.H.G., BARNARD, A.L., WILLIAMS, M., PAGE, M., LING, C., STOTT, E.J., SILVERA, P., TAFFS, L.F., KINGSMAN, A.S., ADAMS, S.E., ALMOND, N., KITCHIN, P.A., AND JONES, W.C. (1991). Vaccine induced $CD4^+$ T cells against the simians immunodeficiency virus *gag* protein. *J. Immunol.* **147**, 3560-3567.

MILLS, K.H.G., PAGE, M, CHAN, W.L., KITCHIN, P., STOTT, E.J., TAFFS, L.F., JONES, W., ROSE, J., LING, C., SILVERA, P., CORCORAN, T., FLANAGAN, B., BURNY, A., BEX, F., DELCHAMBRE, M., VAN OPSTAL, O., FABRY, L., THIRIART, C., DELERS, A., DEWILDE, M., BRUCK, C. (1992). Protection against SIV infection in macaques by immunization with inactivated virus from the BK28 molecular clone but not with BK28-derived recombinant env and gag proteins. *J. Med. Primatol.* **21**. 50-58.

MILLS, K.H.G., PAGE, M., KITCHIN, P., CHAN, W.L., JONES, W., SILVERA, P., CORCORAN, T., FLANAGAN, B., LING, C., THIRIART, C., DEWILDE, M., BRUCK, C., RUD, E., CLARKE, B., AND STOTT, E.J. (1993). Immunization of macaques with SIV env recombinants: specificity of T cell and antibody responses and evaluation of protective efficacy. *J. Med. Primatol.* In press.

MISUMI, Y., MIKI, K., TAKATSUKI, A., TAMURA, G., AND IKEHARA, Y. (1986). Novel blockade by brefeldin-A of intracellular transport of secretory proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261**, 1398-1403.

MITSUYA, H., WEINHOLD, K.J., FURMAN, P.A., STCLAIR, M.H., LEHRMAN, S.N., GALLO, R.C., BOLOGNESI, D., BARRY, D.W., AND BRODER, S. (1985). 3'-azido-3'-deoxythymidine (BW A509U) - an antiviral agent that inhibits the infectivity and cytopathic effect of human lymphotropic T virus type III lymphadenopathy-associated virus *in vitro*. *Proc. Natl. Acad. Sci. USA* **82**, 7096-7100.

MONACO, J.J., AND MCDEVITT, H.O. (1982). Identification of a fourth class of proteins linked to the murine major histocompatibility complex. *Proc. Natl. Acad. Sci. USA* **79**, 3001-3005.

MONACO, J.J., AND MCDEVITT, H.O. (1984). H-2-linked low-molecular weight polypeptide antigens assemble into an unusual macromolecular complex. *Nature* **309**, 797-799.

MONACO, J.J., AND MCDEVITT, H.O. (1986). The LMP antigens: a stable MHC-controlled multisubunit protein complex. *Hum. Immunol.* **15**, 416-426.

MONACO, J.J., CHO, S., AND ATTAYA, M. (1990). Transport protein genes in the murine MHC: possible implications for antigen processing. *Science* **259**, 1723-1726.

MOORE, M.W., CARBONE, F.R., AND BEVAN, M.J. (1988). Introduction of soluble protein into class I pathway of antigen processing and presentation. *Cell* **54**, 777-785.

MOREIN, B., SUNDQUIST, B., HOGLUND, S., DALSGAARD, K., AND OSTERHANS, A. (1984). Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. *Nature* **308**, 457-460.

MORENO, J., VIGNALI, D.A.A., NADIMI, F., FUCHS, S., ADORINI, L., AND HAMMERLING, G.J. (1991). Processing of an endogenous protein can generate MHC class II-restricted T cell determinants distinct from those derived from exogenous antigen. *J. Immunol.* **147**, 3306-3310.

MORIKAWA, Y., BARSOV, E., AND JONES, I. (1993). Legitimate and illegitimate cleavage of human immunodeficiency virus glycoproteins by furin. *J. Virol.* **67**, 3601-3604.

MOWAT, A.M., DONACHIE, A.M., REID, G., AND JARRETT, O. (1991). Induction of class I MHC-restricted T cells and stimulation of mucosal immunity by incorporation of soluble protein in ISCOMs. Proceedings from*Lymhphoid Cells and Molecules in Infection Immunity, Statens Seruminstitut, Elsinore, Denmark.* p33.

MULLIGAN, R.C., AND BERG, P. (1981). Selection for animal cells that express the *E. coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**, 2072-2076.

MURPHY-CORB, M., MARTIN, L.N., DAVISON-FAIRBURN, B., MONTELARO, C., MILLER, M., WEST, M., OHKAWA, S., BASKIN, G.B., ZHANG, J.-Y., PUTNEY, S.D., ALLISON, A.C., AND EPPSTEIN, D.A. (1989). A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**, 1293-1297.

NABEL, E.G., PLAUTZ, G., AND NABEL, G.J. (1990). Site-specific gene transfer *in vivo* by direct gene transfer into the arterial wall. *Science* **249**, 1285-1288.

NEEFJES, J.J., AND PLOEGH, H.L. (1988). Allele- and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with b_2-microglobulin: differential effects of inhibition of glycosylation on class I association. *Eur. J. Immunol.* **18**, 801-810.

NELSON, C.A., ROOF, R.W., MCCOURT, D.W., AND UNANUE, E.R. (1992). Identification of the naturally processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule I-A^k. *Proc. Natl. Acad. Sci. USA* **89**, 7380-7383.

OHASHI, P.S., OEHEN, S., BUERKI, K., PIRCHER, H., OHASHI, C.T., ODERMATT, B. , MALISSEN, B., ZINKERNAGEL, R.M., AND HENGARTNER, H. (1991). Ablation of 'tolerance' and induction of diabetes by virus infection in varal antigen transgenic mice. *Cell* **65**, 305-317.

OLDSTONE, M.B.A. (1993). Transgenic mice as models for studying viral pathogenesis. Proceedings from *The IXth International Congress of Virology, Glasgow, U.K.*, p. 8.

OLDSTONE, M.B., NERENBERG, M., SOUTHERN, P., PRICE, J., AND LEWICKI, H. (1991). Virus infection triggers insulin-dependent *Diabetes mellitus* in transgenic model: role of anti-self (virus) immune response. *Cell* **65**, 319-331.

O'REILLY, D.R., MILLER, L.K., AND LUCKOW, V.A. (1992). *Baculovirus Expression Vectors, a Laboratory Manual.* W. H. Freeman and Company, New York.

ORLANDI, R., GÜSSOW, D.H., JONES, P.T., AND WINTER, G. (1989). Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86**, 3833-3837.

OROSZLAN, S., AND LUFTIG, R.B. (1990). Retroviral proteinases. In *Current Topics in Immunology* , edited by Campans, R.W., Cooper, M., Koprowski, H., Meichers, F., Nussenz-Vogt, P.K., Wagner, H., and Wilson, I., Spring-Verlag, Berlin.

O'SULLIVAN, D., SIDNEY, J., ARRHENIUS, T., DEL GUERCIO, M.F., ALBERTSON, M., WALL, M., SOUTHWOOD, S., COLON, S.M., GAETA, F.C.A., AND SETTE, A. (1991). On the interaction of promiscuous antigenic peptides with different DR alleles. The identification of common structural motifs. *J. Immunol.* **147**, 2663-2669.

PAABO, S., NILSSON, T., AND PETERSON, P.A. (1986) Adenoviruses of subgenera B, C, D and E modulate cell-surface expression of major histocompatibility complex class I antigens. *Proc. Natl. Acad. Sci. USA* **83**, 9665-9669.

PACK, P., KUJAU, M., SCHROECKH, V., KNUPFER, U., WENDEROTH, R., RIESENBERG, D., AND PLUCKTHUN, A. (1993). Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high-cell density fermentation of *Escherichia coli. Biotechnology* **11**, 1271-1277.

PAGE, M.J., AND SYDENHAM, M.A. (1991). High level expression of the humanized monoclonal antibody CAMPATH-1H in Chinese hamster ovary cells. *Biotechnology* **9**, 64-68.

PANTALEO, G., GRAZIOSI, C., DEMAREST, J.F., BUTINI, L., MONTRONI, M., FOX, C.H., ORENSTEIN, J.M., KOTLER, D.P., AND FAUCI, A.S. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **263**, 355-358.

PAUWELS, R., ANDRIES, K., DESMYTER, J., SCHOLS, D., KUKLA, M.J., BRESLIN, H.J., RAEYMAECKERS, A., VANGELDER, J., WOESTENBORGHS, R., HEYKANTS, J., SCHELLEKENS, K., JANSEN, M.A.C., DECLERQ, E., AND JANSSEN, P.A.J. (1990). Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives. *Nature* **343**, 470-474.

PHILLIPS, R.E., ROWLAND-JONES, S., NIXON, D.F., GOTCH, F.M., EDWARDS, J.P., OGUNLESI, A.O., ELVIN, J.G., ROTHBARD, J.A., BANGHAM, C.R.M., RIZZA, C.R., AND MCMICHAEL, A.J. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **254**, 453-459.

PITHA, P.M., WIVEL, N.A., FERNIE, B.F., AND HARPER, H.P. (1979). Effect of interferon on murine leukaemia virus infection. IV. Formation of non-infectious virus in chronically-infected cells. *J. Gen. Virol.* **42**, 467-470.

POPOVIC, M., SARNGADHARAN, M., READ, E., AND GALLO, R.C. (1984). Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**, 497-500.

POWIS, S.J., DEVERSON, E.V., COADWELL, W.J., CIRUELA, A., HUSKISSON, N.S., SMITH, H., BUTCHER, G.W., AND HOWARD, J.C. (1992). Effect of a polymorphism of an MHC-linked transporter on the peptide assembled in class I molecules. *Nature* **357**, 211-215.

PULLEN, A.M., WADE, T., MARRACK, P., AND KAPPLER, J.W. (1990). Identification of the region of T cell receptor beta chain that interacts with the self-superantigen Mls-1a. *Cell* **61**, 1365-1374.

PURVIS, I.J., BETHANY, A.J.E., SANTIAGO, T.C., COGGINS, J.R., DUNCAN, K., EASON, R., AND BROWN, A.J.P. (1987). The efficiency of folding of some proteins is increased by a controlled rates of translation *in vivo*: a hypothesis. *J. Mol. Biol.* **193**, 413-417.

PUTKONEN, P., THORSTENSSON, R., ALBERT, J., HILD, K., NORRBY, E., BIBERFELD, P., AND BIBERFELD, G. (1990). Infection of cynomolgus monkeys with HIV-2 protects against pathogenic consequences of a subsequent simian immunodeficiency virus infection. *AIDS* **4**, 783-789.

PUTKONEN, P., THORSTENSSON, R., WALTHER, L., ALBERT, J., AKERBLOM, L., GRANQUIST, O., WADELL, G., NORRBY, E., AND BIBERFELD, G. (1991). Vaccine protection against HIV-2 infection in cynomolgus monkeys. *AIDS Res. Hum. Retro.* **7**, 271-277.

RAMMENSEE, H.-G., FALK, K., AND ROTZSCHKE, O. (1993). Peptides naturally presented by MHC class I molecules. *Annu. Rev. Immunol.* **11**, 213-244.

RANDALL, R.E. (1989). Soild matrix-antibody-antigen (SMAA) complexes for constructing multivalent subunit vaccines. *Immunol. Today* **10**, 336-339.

RANDALL, R.E., AND SOUBERBIELLE, B.E. (1990). Presentation of virus antigens for the induction of protective immunity. In *Control of Virus Diseases*, edited by N.J. Dimmock, P.D. Griffiths and C.R. Madeley, Cambridge, Cambridge University Press. pp. 21-51.

RANDALL, R.E., AND YOUNG, D.F. (1988). Humoral and cytotoxic T cell responses to internal and external structural proteins of simian virus 5 by immunization with solid matrix-antibody-antigen complexes. *J. Gen. Virol.* **69**, 2505-2516.

RANDALL, R.E., AND YOUNG, D.F. (1989). Immunization against multiple viruses using solid matrix-antibody-antigen complexes. *J. Virol.* **63**, 1808-1810.

RANDALL, R.E., AND YOUNG, D.F. (1991). Solid matrix-antibody-antigen complexes induce antigen-specific $CD8^+$ cells that clear a persistent paramyxovirus infection. *J. Virol.* **65**, 719-726.

RANDALL, R.E., YOUNG, D.F., GOSWAMI, K.K.A., AND RUSSELL, W.C. (1987). Isolation and characterization 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.

RANDALL, R.E., HANKE, T., YOUNG, D., AND SOUTHERN, J.A. (1993a). Two-tag purification of recombinant proteins for the construction of solid matrix-antibody-antigen (SMAA) complexes as vaccine. *Vaccine* **11**, 1247-1252.

RANDALL, R.E., YOUNG, D., HANKE, T., SZAWLOWSKI, P. AND BOTTING, C. (1993b). Two-tag affinity purification of antibody-antigen complexes containing SIV proteins; comparison of antigen and antibody-antigen complexes for immune priming. *Vaccine* In press.

RAUH, L.W., AND SCHMIDT, R. (1965). Measles immunization with killed virus vaccines. *American J. Dis. Children* **109**, 232-237.

RAWLE, F.C., TOLLEFSON, A.E., WOLD, W.S.M., AND GOODING, L.R. (1989). Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. *J. Immunol.* **143**, 2031-2037.

RAY, C.A., BLACH, R.A., KRONHEIM, S.R., GREENSTREET, T.A., SLEATH, P.R., SALVESEN, G.S., AND PICKUP, D.J. (1992). Viral inhibition of inflamation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell* **69**, 97-604.

REDDEHASE, M.J., ROTHBARD, J.B., AND KOSZINOWSKI, U.H. (1989). A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes. *Nature* **337**, 651-653.

REICHMANN, L., CLARK, M., WALDMANN, H., AND WINTER, G. (1988). Reshaping human antibodies for therapy. *Nature* **332**, 323-327.

ROBERTS, T.M., KACICH, R., AND PTASCHE, M. (1979). A general method for maximizing the expression of a cloned gene. *Proc. Natl. Acad. Sci. USA* **76**, 760-764.

ROBERTS, N.A., MARTIN, J.A., KINCHINGTON, D., BROADHURST, A.V., CRAIG, J.C., DUNCAN, I.B., GALPIN, S.A., HANDA, B.K., KAY, J., KROHN, A., LAMBERT, R.W., MERRETT, J.H., MILLS, J.S., PARKES, K.E.B., REDSHAW, S., RITCHIE, A.J., TAYLOR, D.L., THOMAS, G.J., AND MACHIN, P.J. (1990). Rational design of peptide-based HIV proteinase inhibitors. *Science* **248**, 358-361.

ROBINSON, M., LILLEY, R., LITTLE, S., EMTAGE, J.S., YARRANTON, G., STEPHENS, P., MILLICAN, A., EATON, M., AND HUMPHREYS, G. (1984). Codon usage can affect efficiency of translation of genes in *E. coli*. *J. Bacteriol.* **171**, 4617-4622.

ROCHE, P.A., AND CRESSWELL, P. (1991a). High-affinity binding of an influenza haemagglutinin-derived peptide to purified HLA-DR. *J. Immunol.* **144**, 1849-1856.

ROCHE, P.A., AND CRESSWELL, P. (1991b). Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl. Acad. Sci. USA* **88**, 3150-3154.

ROCHE, P.A., MARKS, M.S., AND CRESSWELL, P. (1991). The HLA class II-invariant chain complex is a nine-subunit transmembrane glycoprotein. *Nature* **354**, 392-394.

ROMERO, D.L., BUSSO, M., TAN, C.K., REUSSER, F., PALMER, J.R., POPPE, S.M., ARISTOFF, P.A., DOWNEY, K.M., SO, A.G., RESNICK, L., AND TARPLEY, W.G. (1991). Non-nucleoside reverse transcriptase inhibitors that potently and specifically block human immunodeficiency virus type 1 replication. *Proc. Natl. Acad. Sci. USA* **88**, 8806-8810.

ROOF, R.W., LEUSCHER, I.F., AND UNANUE, E.R. (1990). Phospholipids enhance the binding of peptides for class II major histocompatibility molecules. *Proc. Natl. Acad. Sci. USA* **87**, 1735-1739.

ROTHBARD, J.B., ND TAYLOR, W.R. (1988). A sequence pattern common to T cell epitopes. *EMBO J.* **7**, 93-100.

ROTZSCHKE, O., FALK, K., DERES, K., SCHILD, H., NORDA, M., METZGER, J., JUNG, G., AND RAMMENSEE, H.G. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* **348**, 252-254.

ROTZSCHKE, O., FALK, K., FAATH, S., AND RAMMENSEE, H.G. (1991). On the nature of peptides involved in T cell alloreactivity. *J. Exp. Med.* **174**, 1059-1071.

ROTZSCHKE, O., FALK, K., STEVANOVIC, S., GRAHOVAC, B., SOLOSKI, M.J., JUUG, G., AND RAMMENSEE, H.G. (1993). Qa-2 molecules are peptides receptors of higher stringency than ordinary class I molecules. *Nature* **361**, 642-644.

RUD, E.W. (1991). SIVmac32(pJ5) sequence. DDBJ, EMBL and GENBANK entry number D01065. Also submitted to the Los Alamos HIV database and published in the 1991 Fall update of the database.

RUD, E.W., YON, J.R., LADER, B.A., COOK, N., CRANAGE, M.P., AND CLARKE, B.E. (1992). Infectious molecular clones of SIVmac32H: nef deletion controls ability to reisolate virus from rhesus macaque. In *Vaccine 92: Modern Approaches to New Vaccines including AIDS*. Edited by F. Brown, R.M. Chanock, H.S. Ginsberg and R.A. Lerher. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 239-245.

RUDENSKY, A.Y., PRESTON-HURLBURT, P., HONG, S.-C., BARLOW, A., AND JANEWAY, C.A. (1991a). Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353**, 622-627.

RUDENSKY, A.Y., RATH, S., PRESTON-HURLBURT, P., MURPHY, D.B., AND JANEWAY, C.A. (1991b). On the complexity of self. *Nature* **353**, 660-662.

SAARI, W.S., SCHWERING, J.E., LYLE, P.A., ENGELHARDT, E.L., SARTORELLI, A.C., AND ROCKWELL, S. (1991) Synthesis and evaluation of some nitrobenzensulfonamids containing nitroisopropyl and (ureidooxy)methyl groups as novel hypoxic cell selective cytotoxic agents. *J. Med. Chem.* **34**, 2922-2926.

SADEGH-NASSERI, S., AND GERMAIN, R.N. (1991). A role for peptide in determining MHC class II structure. *Nature* **353**, 167-170.

SAKURAGI, S., SHIBATA, R., MUKAI, R., KOMATSU, T., FUKASAWA, M., SAKAI, H., SAKURAGI, J.-I., KAWAMURA, M., IBUKI, K., HAYAMI, M., AND ADACHI, A. (1992). Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus. *J. Gen. Virol.* **73**, 2983-2987.

SASSENFELD, H.M. (1990). Engineering proteins for purification. *Trends in Biotechnol.* **8**, 88-93.

SCHOLL, P., DIEZ, A., AND GEHA, R.S. (1989). Staphylococcal enterotoxin B and toxic shock syndrome toxin-1 bind to distinct sites on HLA-DR and HLA-DQ molecules. *J. Immunol.* **143**, 2583.

SCHONRICH, G., KALINKE, U., MOMBURG, F., MALISSEN, M., SCHMITT, V.A., MALISSEN, B., HAMMERLING, G.J., AND ARNOLD, B. (1991). Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* **65**, 293-304.

SCHRIER, P.I., BERNARDS, R., VAESSEN, R.T.M.J., HOUWELING, A., AND VAN DER EB, A.J. (1983). Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature* **305**, 771-775.

SCHULTZ, A.M., AND HU, S.-I. (1993). Primate models for HIV vaccines. *AIDS* **7**, (suppl 1), S161-S170.

SCHULZ, M., AICHELE, P., SCHNEIDER, R., HANSEN, T.H., ZINKERNAGEL, R.M., AND HENGARTNER, H. (1991). Major histocompatibilty complex binding and T cell recognition of a viral nonapeptide containing a minimal tetrapeptide. *Eur. J. Immunol.* **21**, 1181-1185.

SCHWARTZ, S., FELBER, B.K., FENYO, E.-M., AND PAVLAKIS, G.N. (1990). Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. *J. Virol.* **64**, 5448-5456.

SCHWARTZ, D.H., SKOWRON, G., AND MERIGAN, T.C. (1991). Safety and effects of interleukin-2 plus zidovudine in asymptomatic individuals infected with human immunodeficiency virus. *J. Acquir. Immune Defic. Syndr.* **4**, 11-23.

SCOTT, P. (1993). Selective differentiation of CD4+ T helper cell subsets. *Curr. Opin. Immunol.* **5**, 391-397.

SEKALY, R.-P., CROTEUA, G., BOWMAN, M., SCHOLL, P., BURAKOFF, S., AND GEHA, R.S. (1991). The CD4 molecule is not always required for the T cell response to bacterial enterotoxins. *J. Exp. Med.* **173**, 367-371.

SETTE, A., BUUS, S., COLON, S., SMITH, J.A., MILES, C., AND GREY, H.M. (1987). Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* **328**, 395-399.

SHAFFERMAN, A., JAHRING, P.B., BENVENISTE, R.E., LEWIS, M.G., PHIPPS, T.J., EDIN-MCCUTCHAN, F., SADOFF, J., EDDY, G.A., AND BURKE, D.S. (1991). Protection of macaques with an SIV envelope peptide vaccine based on conserved HIV-1 sequences. *Proc. Natl. Acad. Sci. USA* **88**, 7126-7130.

SHAFFERMAN, A., LEWIS, M.G., MCCUTCHAN, F.E., BENVENISTE, R.E., JAHRING, P.B., BURKE, D.S. AND, EDDY, G.A. (1992). Prevention of transmission of simian immunodeficiency virus from vaccinated macaques that developed transient virus infection following challenge. *Vaccine* **11**, 848-853.

SHAWAR, S.M., VYAS, J.M., RODGERS, J.R., COOK, R.G., AND RICH, R.R. (1991). Specialized functions of MHC class I molecules. II. Hmt binds N-formylated peptides of mitochondrial and prokaryotic origin. *J. Exp. Med.* **174**, 941-944.

SHEARER, G.M., AND CLERICI, M. (1993). Protective cell-mediated immunity for HIV infection/AIDS progression? Proceedings from *MRC AIDS-Directed Programme Workshop, Brighton, U.K.*, p. 100.

SHIBATA, R., KAWAMURA, M., SAKAI, H., HAYAMI, M., ISHIMOTO, A., AND ADACHI, A. (1991). Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. *J. Virol.* **65**, 3514-3520.

SILICIANO, R.F., LAWTON, T., KNALL, C., KARR, R.W., BERMAN, P., GREGORY, T., AND REINHERZ, E.L. (1988). Analysis of host-virus interactions in AIDS with anti-gp120 T cell clones: effect of HIV sequence variation and a mechanism for $CD4^+$ cell depletion. *Cell* **54**, 561-575.

SILVER, M.L., GUO, H.-C., STROMINGER, J.L., AND WILEY, D.C. (1992). Atomic structure of a human MHC molecule presenting an influenza virus peptide. *Nature* **360**, 367-369.

SIMON, L.D., RANDOLPH, B., IRWIN, N., AND BINKOWSKI, G. (1983). Stabilization of proteins by a bacteriophage T4 gene cloned in *E. coli*. *Proc. Natl. Acad. Sci. USA* **80**, 2059-2062.

SJODAHL, J. (1976). Repetitive sequences in protein A from *Staphylococcus aureus*: three highly homologous Fc-binding regions. *FEBS Lett.* **67**, 62-65.

SJOHOLM, I. (1975). Protein A from *Staphylococcus aureus*. *Eur. J. Biochem.* **51**, 55-58.

SJOQUIST, J., MOVITZ, J., JOHANSSON, I.-B., AND HJELM, H. (1972). Localization of protein A in the bacteria. *Eur. J. Biochem.* **30**, 190-194.

SKERRA, A., PFITZINGER, I., AND PLUCKTHUN, A. (1991). The functional expression of antibody Fv fragment in *Escherichia coli*: improved vectors and generally applicable purification technique. *Bio/Technology* **9**, 273-278.

SMITH, G.L., AND CHAN, Y.S. (1991). Two vaccinia virus proteins structurally related to the interleukin-1 receptor and the immunoglobulin superfamily. *J. Gen. Virol.* **72**, 511-518.

SMITH, D.B., AND JOHNSON, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-*S*-transferase. *Gene* **67**, 31-40.

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., HOWARD, S.T. AND CHAN, Y.S. (1989). Vaccinia virus encodes a family of genes with homology to serine protease inhibitors. *J. Gen. Virol.* **70**, 2333-2343.

SOUDEYNS, H., PANTALEO, G.P., BOGHOSSIAN, T., CIURLI, C., REBAI, N., LANE, H.C., FAUCI, A.S., AND SEKALY, R.P. (1993). V-beta-specific variations in the TCR repertoir of HIV-infected subjects suggest the presence of an HIV-associated superantigen. *J. Immunol.* **150**. 104-108.

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.

SPIES, T., AND DEMARS, R. (1991). Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. *Nature* **351**, 323-324.

SPRINGER, T.A. (1990). Adhesion receptors of the immune system. *Nature* **346**, 425-434.

STAERZ, U.D., KARASUYAMA, H., AND GAMER, A.M. (1987). Cyto-toxic lymphocytes-T against a soluble protein. *Nature* **329**, 449-451.

STANLEY, S.K., AND FAUCI, A.S. (1993). T-cell homeostasis in HIV infection - part of the solution, part of the problem. *J. Acquired Immune Defic. Syndr.* **6**, 142-143.

STEIN, B.S., GOWDA, S.F., LIFSON, J.D., PENHALLOW, R.C., BENSCH, K.G., AND ENGELMAN, E.G. (1987). pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* **49**, 659-668.

STOTT, E.J. (1993). Towards a vaccine against AIDS - lessons from simian immunodeficiency virus vaccines. In press

STOTT, E.J., CHAN, W.L., MILLS, K.H.G., PAGE, M., TAFFS, F., CRANAGE, M., GREENAWAY, P., AND KITCHIN, P.A. (1990). Preliminary report: protection of cynomolgus macaques against simian immunodeficiency virus by a whole fixed cell vaccine. *Lancet* **336**, 1538-1541.

STOTT, E.J., KITCHIN, P.A., PAGE, M., FLANAGAN, B., TAFFS, L.F., CHAN, W.L., MILLS, K.H.G., SILVERA, P., AND RODGER, A., (1991). Anti-cell antibody in macaques. *Nature* **353**, 393.

STUDIER, F.W., ROSENBERG, A.H., DUNN, J.J., AND DUBENDORFF, J.W. (1993). Used of T7 polymerase to direct the expression of cloned genes. *Methods Enzymol.* **185**, 60-89.

SULLENGER, B.A., GALLARDO, H.F., UNGERS, G.E., AND GILBOA, E. (1991a). Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. *Cell* **63**, 601-608.

SULLENGER, B.A., GALLARDO, H.F., UNGERS, G.E., AND GILBOA, E. (1991b). Analysis of trans-acting response decoy RNA-mediated inhibition of human immunodeficiency virus type 1 transactivation. *J. Virol.* **65**, 6811-6816.

SUTJIPTO, S., PEDERSEN, N.C., MILLER, C.J., GARDNER, M.B., HANSON, C.V., GETTIE, A., JENNINGS, M., HIGGINS, J., AND MARX, P.A. (1990). Inactivated simian immunodeficiency virus vaccine failed to protect rhesus macaques from intravenous or genital mucosal infection but delayed disease in intravenously exposed animals. *J. Virol.* **64**, 2290-2297.

SWANSTROM, R., KAPLAN, A.H., AND MANCHESTER, M. (1990). The aspartic proteinase of HIV-1. *Semin. Virol.* **1**, 175-186.

SZAWLOWSKI, P.W.S., HANKE, T., AND RANDALL, R.E. (1993). Sequence homology between HIV-1 gp120 and the apoptosis-mediating protein Fas. *AIDS* **7**, 1018.

TAKAHASHI, N., VEDA, S., OBATU, M., NIKAIDO, T., NAKAI, S., AND HONJO, T. (1982). Structure of human immunoglobulin gamma genes: implications for evolution of a gene family. *Cell* **29**, 671-679.

TAKAHASHI, H., TAKESHITA, T., MOREIN, B., PUTNEY, S., GERMAIN, R.N., AND BERZOFKY, J.A. (1990). Induction of $CD8^+$ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. *Nature* **344**, 873-875.

TAN, C.-K., CIVIL, R., MIAN, A.M., SO, A.G., AND DOWNEY, K.M. (1991). Inhibition of the RNase H activity of HIV reverse transcriptase by zidothymidylate. *Biochemistry* **30**, 4831-4835.

TEMPEST, P.R., BREMNER, P., LAMBERT, M., TAYLOR, G., FURZE, J.M., CARP, F.J., AND HARRIS, W.J. (1991) Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection *in vivo*. *Biotechnology* **9**, 266-271.

TERSMETTE, M., VANDONGEN, J.J.M., CLAPHAM, P.R., DEGOEDE, R.E.Y., WOLVERSTETTERO, I.L.M., VANKESSEL, A.G., HUISAN, J.G., WEISS, R.A., AND MIEDEMA, F. (1989a). Human immunodeficiency virus infection studied in CD4-expressing human murine T cell hybrids. *Virology* **168**, 267-273.

TERSMETTE, M., GRUTERS, R.A., DEWOLF, F., DEGOEDE, R.E.Y., LANGE, J.M.A., SCHELLEKENS, P.T.A., GOUDSMIT, J., HUISMAN, H.G., AND MIEDEMA, F. (1989b). Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome - studies on sequential HIV isolates. *J. Virol.* **63**, 2118-2125.

TEYTON, L., O'SULLIVAN, D., DICKSON, P.W., LOTTEUA, V., SETTE, A., FINK, P., AND PETERSON, P.A. (1990). Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature* **348**, 39-44.

THOMASON, D.B., AND BOOTH, F.W. (1990). Stable incorporation of a bacterial gene into adult rat sceletal muscle *in vivo*. *Amer. J. Physiol.* **27**, C578-C581.

TICE, T.R. (1993). Oral, parenteral and intranasal vaccine delivery using biodegradable poly(lactide-co-glycolide) microspheres. Proceedings from*Novel Vaccines Strategies, Holiday Inn Bethesda, Bethesda, MD, USA.*

TONG-STARKSEN, S., LUCIW, P.A., AND PETERLIN, B.M. (1989). Human immunodeficiency virus long terminal repeat responds to T cell activation signals. *Proc. Natl. Acad. Sci. USA* **87**, 7861-7865.

TOWNSEND, A.R.M., MCMICHAEL, A.J., CARTER, N.P., HUDDLESTON, J.A., AND BROWNLEE, G.G. (1984). Cytotoxic T cell recognition of the influenza nucleoprotein and haemagglutinin expressed in transfected mouse L cells. *Cell* **39**,13-25.

TOWNSEND, A.R.M., GOTCH, F.M., AND DAVEY, J. (1985). Cytotoxic T cells recognize fragments of influenza nucleoprotein. *Cell* **42**, 457-467.

TOWNSEND, A., ROTHBARD, J., GOTCH, F., BAHADUR, B., AND WRAITH, D. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell* **44**, 959-968.

TOWNSEND, A., BASTIN, J., GOULD, K., BROWNLEE, G., ANDREWS, M., COUPAR, B., BOYLE, D., CHAN, S., AND SMITH, G. (1988). Defective presentation to class I-restricted cytotoxic T lymphocytes in vaccinia-infected cells is overcome by enhanced degradation of antigen. *J. Exp. Med.* **168**, 1211-1224.

TOWNSEND, A., OHLEN, C., FOSTER, L., BASTIN, J., LJUNGGREN H.-G., AND KARRE, K. (1989). A mutant cell in which association of class I heavy and light chains is induced by viral peptides. *Cold Spring Harbor Symp. Quant. Biol.* **54**, 299-308.

TOWNSEND, A., ELLIOTT, T., CERUNDOLO, V., FOSTER, L., BARBER, B., AND TSE, A. (1990). Assembly of MHC class I molecules analyzed *in vitro*. *Cell* **62**, 285-295.

TREDE, N., GEHA, R.S., AND CHATILA, T. (1991). Transcriptional activation of monokine genes by MHC class II ligands. *J. Immunol.* **146**, 2310-2315.

TROWSDALE, J., HANSON, I., MOCKRIDGE, I., BECK, S., TOWNSEND, A., AND KELLY, A. (1990). Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. *Nature* **348**, 741-744.

TSUJIMOTO, H., COOPER, R.W., KODAMA, T., FUKASAWA, M., MIURA, T., OHTA, Y., ISHIKAWA, K.I., NAKAI, M., FROST, E., ROELANTS, G.E., ROFFI, J., AND HAYAMI, M. (1988). Isolation and characterisation of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. *J. Virol.* **62**, 4044-4050.

VALENZUELA, P.A., 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 BLEEK, G.M., AND NATHENSON, S.G. (1990). Isolation of an immunodominant viral peptide from class I H-2K^b molecule. *Nature* **348**, 213-216.

VANDEVELDE, H., VANHOEGEN, I., LUO, W., PARNES, J.R., AND THIELEMANS, K. (1991). The B cell surface protein CD27/Lyb-2 is the ligand for CD5. *Nature* **351**, 662-665.

VAN NOORT, J.M., BOON, J., VAN DER DRIFT, A.C.M., WAGENAAR, J.P.A., BOOTS, A.M.H., AND BOOG, C.J.P. (1991). Antigen processing by endosomal proteases determines which sites of sperm-whale myoglobin are eventually recognized by T cells. *Eur. J. Immunol.* **21**, 1989-1996.

VAN REGENMORTEL, M.H.V. (1989) Structural and functional approaches to the study of protein antigenicity. *Immunol. Today* **10**, 266-272.

VARMUS, H.E., AND SWANSTROM, R. (1985). Replication of retroviruses. In *RNA Tumor Viruses,* edited by R. Weiss, N. Teich, H. Varmus and J. Coffin, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 75-134.

VERHOEYEN, M., MILSTEIN, C., AND WINTER, G. (1988). Reshaping human antibodies: grafting an antilysozyme activity. *Science* **239**:1534-1536.

VERONESE, P.D.M., COPELAND, T.D., OROSZLAN, S., GALLO, R.C., AND SARNGADHARAN, M.G. (1988). Biochemical and immunological analysis of human immunodeficiency virus gag gene products p17 and p24. *J. Virol.* **62**, 795-801.

VIDOVIC, D., AND MATZINGER, P. (1988). Unresponsiveness to a foreign antigen can be caused by self-tolerance. *Nature* **336**, 222-225.

VILLAREJIO, M.R., AND ZABIN, I. (1974). Beta-galactosidase from termination and deletion mutant strains. *J. Bacteriol.* **110**, 171-178.

WAHREN, B., ROSEN, J., SANDSTROM, E., MATHIESEN, T., MODROW, S. AND WIGZELL, H. (1989). HIV-1 peptides induce a proliferative response in lymphocytes from infected persons. *J. AIDS* **4**, 448.

WALLNY, H.-J., ROTZSCHKE, O., FALK, K., HAMMERLING, G., AND RAMMENSEE, H-G. (1992). Gene transfer experiments imply instructive role of MHC class I molecules in cellular peptide processing. *Eur. J. Immunol.* **22**, 655-659.

WANG, B., UGEN, K.E., SRIKANTAN, V., AGADJANYAN, M.G., DANG, K., REFAELI, Y., SATO, A.I., BOYER, J., WILLIAMS, W.V., AND WEINER, D.B. (1993). Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**, 4156-4160.

WATARI, E., DIETZSCHOLD, B., SZOKAN, G., AND HEBER-KATZ, E. (1987). A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. *J. Exp. Med.* **165**, 459-470.

WEBER, S., TRAUNECKER, A., OLIVERI, F., GERHARD, W., AND KARJALAINEN, K. (1992). Specifc low-affinity recognition of major histicompatibilty complex plus peptide by soluble T cell receptor. *Nature* **356**, 793-795.

WEI, M.I., AND CRESSWELL, P. (1992). HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443-446.

WEINHOLD, K.J., LYERLY, H.K., STANLEY, S.D., AUSTIN, A.A., MATTHEWS, T.J., AND BOLOGNESI, D.P. (1989). HIV-1 gp120-mediated immune suppression and lymphocyte destruction in the absences of viral infection. *J. Immunol.* **142**, 3091-3097.

WEISS, R.A. (1993). How does HIV cause AIDS? *Science* **260**, 1273-1279.

WEISS, S., AND BOGEN, B. (1989). B lymphoma cells process and present their endogenous immunoglobulin to major histocompatibility complex-restricted T cells. *Proc. Natl. Acad. Sci. USA* **86**, 282-286.

WEISS, S., AND BOGEN, B. (1991). MHC class II-restricted presentation of intracellular antigen. *Cell* **64**, 767-776.

WHITE, J., HERMAN, A., PULLEN, A.M., KUBO, R., KAPPLER, J.W., AND MARRACK, P. (1989). The Vβ-specific staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56**, 27-35.

WHITTON, J.L., TISHON, A., LEWICKI, H., GEBHARD, J., COOK, T., SALVATO, M., JOLLY, E., AND OLDSTONE, M.B.A. (1989). Molecular analysis of a five-amino acid cytotoxic T lymphocyte (CTL) epitope: an immunodominat region which induces nonreciprocal CTL cross-reactivity. *J. Virol.* **63**, 4303-4310.

WILLIAMS, A.F., AND BEYERS, A.D. (1992). T cell receptors: at grips with interactions. *Nature* **356**, 746.

WILSON, J.M. (1993). Vehicles for gene therapy. *Nature* **265**, 691-692.

WINTER, G., AND MILSTEIN, C. (1991). Man-made antibodies. *Nature* **349**, 293-299.

WOLFF, J.A., MALONE, R.W., WILLIAMS, P., CHONG, W., ASCADI, G., JANI, A., AND FELGNER, P.L. (1990). Direct gene transfer into mouse muscle *in vivo*. *Science* **249**, 1285-1288.

WOODLAND, D.L., HAPP, M.P., GOLOB, K.J., AND PALMER, E. (1991). An endogenous retrovirus mediating deletion of alpha beta cells? *Nature* **349**, 529-530.

YOUNG, J.W., AND STEINMAN, R.M. (1990). Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of $CD4^+$ helper T cells. *J. Exp. Med.* **171**, 1315-1332.

ZENKE, M., STEINLEIN, P., WAGNER, E., COTTEN, M., BEUG, H., AND BIRNSTIEL, M.L. (1990). Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **87**, 3655-3659.

ZHANG, W., YOUNG , A.C.M., IMARAI, M., NATHESON, S.G., AND SACCHETTINI, J.C. (1992). Crystal structure of the major histocompatibilty complex class I H-$2K^b$ molecule containing a single viral peptide: implication for the peptide binding and T cell recognition. *Proc. Natl. Acad. Sci. USA* **89**, 8403-8407.

ZINKERNAGEL, R.M., AND DOHERTY, P.C. (1975). H-2 compatibility requirement for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* **141**,1427-1436.