INVESTIGATIONS INTO THE COGNITIVE FUNCTIONING OF SUBJECTS WITH EPILEPSY IN RELATION TO ANTICONVULSANT MEDICATION

Kay Garvey

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IMMUNOLOGICAL INVESTIGATION IN MULTIPLE SCLEROSIS

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PhD THESIS

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DECLARATION

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# Table of Contents

## Introduction

1. Background

## Clinical Aspects of MS

3. The Histopathology of MS
   - Location of the MS plaques
   - Perivascular situation of the MS plaques
   - Relapsing-remitting MS and primary progressive MS may be two different diseases
   - Histopathology of the 'active MS plaque'
     - a) The different cell types in the MS plaque - some general considerations
     - b) Histopathological features of the MS lesion
     - c) The oligodendrocytes in the MS plaque
     - d) The astrocytes in the MS plaques
   - The inflammatory cellular infiltrate in the active MS plaque
     - a) The T cell component of the cellular infiltrate
     - b) The B cell component of the cellular infiltrate
     - c) The monocyte-macrophage component of the cellular infiltrate
   - The chronic MS lesion

## The Brain Vessels in MS

16. General consideration: the BBB
   - a) Definition and function of the BBB
   - b) Anatomical structure of the BBB
   - Endothelial cells (EC) and brain endothelial cells
     - a) The endothelial cells and their characterisation
     - b) The brain endothelial cells
   - The brain pericyte and smooth muscle cell
   - Historical background of the brain vessels in relation to MS
Histopathological considerations of the brain vessels in relation to the MS lesions ............................................. 24
Hypotheses: the involvement of the brain blood vessels in the aetiopathogenesis of MS ..................................... 25

THE VIRAL THEORY IN MS .............................................. 28
Epidemiology ...................................................................... 28
Demyelinating diseases caused by viral infections; general consideration .............................................................. 31
Human demyelinating diseases caused by viral infections ....................................................................................... 31
  a) Progressive multifocal leukoencephalopathy .......... 31
  b) Post infectious encephalomyelitis ....................... 33
  c) HTLV-1 .................................................................. 34
  d) Acquired immunodeficiency syndrome (AIDS) .. 35
Viral demyelinating diseases in animals ..................... 37
  a) Theiler's murine encephalomyelitis virus ......... 39
  b) JHM strain of mouse hepatitis virus .......... 40
  c) Canine distemper ............................................. 40
  d) Measles virus (MV) ....................................... 41
  e) Semliki Forest virus (SFV) ......................... 42
  f) Herpes simplex virus type 1 (HSV-1) ......... 42
  g) Visna ................................................................. 43
  h) Caprine arthritis encephalitis ......................... 44
Immune response against viruses in MS ..................... 44
  a) Humoral response ........................................... 44
  b) Cellular immune response .............................. 46
Viral isolates in MS and transmissibility of the disease ......................................................................................... 47
Retroviruses and MS ............................................. 49
Paramyxoviridae and MS ........................................ 51
  a) Measles ............................................................. 52
  b) Canine distemper virus (CDV) ...................... 53
  c) Simian virus 5 (SV5) ..................................... 54
Conclusions on how viruses could be responsible for MS ......................................................................................... 55
  Direct effect of the virus ...................................... 55
  Bystander effect due to viral infection ................. 56
Autoimmunity as a bystander effect of viral infection

a. Release of hidden antigens
b. Presentation of autoantigen through increased expression of HLA molecules
c. Insertion of a viral component into a host component
d. The 'superantigen' hypothesis
e. Incorporation of a host component into a viral component
f. 'Molecular mimicry'
g. Disruption of the immune system regulation

THE CEREBROSPINAL FLUID (CSF) AND MULTIPLE SCLEROSIS

General considerations

a) Circulation of the CSF
b) The composition of the CSF in patients with MS and in normals

Oligoclonal bands and MS

a) Definition and description
b) Other immunoglobulins in the CSF
c) Other diseases show CSF oligoclonal pattern
d) Techniques used to visualise oligoclonal bands
e) Specificity of the oligoclonal IgG in MS

Other components studied in the CSF in relation to MS

IMMUNOLOGY OF MULTIPLE SCLEROSIS

Peripheral blood and CSF immune abnormalities in MS

Studies on a possible autoantigen in MS

a) Antigens of the myelin sheath
b) T cell autoantigens
c) Fine specificity of MBP-specific T cells in patients with MS
d) B cell autoantigens
e) Autoimmunity to brain vessels in MS
Study of experimental autoimmune encephalomyelitis (EAE) and possible therapeutic intervention in MS

CONCLUDING NOTES ON MULTIPLE SCLEROSIS

BACKGROUND TO THIS THESIS

MATERIAL AND METHODS

MM1. Polyacrylamide isoelectric focusing of CSF

MM2. Silver staining of IEF gel or SDS PAGE gels

MM3. Immunoprecipitation of CSF

MM4. Immunoblotting method on agarose gel

MM5. Imprint immunofixation (IIF) method

MM6. Preparation of virus infected cells and immunofluorescence slides

MM7. Indirect immunofluorescence technique

MM8. Tissue culture and virus preparation

MM9. SDS PAGE electrophoresis

MM10. Western blotting protocol

MM10'. Dot blot for protein concentration

MM11. T cell proliferation assay

MM12. Nitrocellulose T cell technique protocol

A/ Spotting technique

B/ The blotting technique

MM13. Statistics

MM14. Myelin preparation

MM15. Brain vessel preparation

MM16. Human brain endothelial cell in culture

MM17. Umbilical cord endothelial cell culture

MM18. Culture of human astrocyte

MM19. g-Glutamyl Transpeptidase assay

MM20. Scanning electron microscopy (SEM) of the brain vessels preparation

MM21. Transmission electron microscopy of the myelin preparation

MM22. Clinical study using the western blotting assay and myelin and human brain vessels as antigens

MM23. Clinical study using the 'nitrocellulose T cell assay' and myelin and human brain vessels as antigens
RESULTS ..................................................................................... 144

R1- PARAMYXOVIRUS SV5 RELATED CSF STUDIES........ 144
R2- ASSESSMENT OF THE TECHNIQUES USED IN THE
STUDIES OF THE POSSIBLE INVOLVEMENT OF SV5 IN
MS: THE IMMUNOABSORPTION, IMMUNOPRINTING
AND THE IMMUNOBLOTTING OF CSF.............................. 157
   a) Sensitivity of the techniques.................................. 159
   b) The specificity of the techniques.......................... 164
SUMMARY AND CONCLUSIONS OF R1 AND R2
SECTIONS............................................................................... 167

R3- PURIFICATION OF MYELIN FROM HUMAN
BRAINS.................................................................................... 168
R4- PURIFICATION OF BRAIN VESSELS FROM BOVINE
AND HUMAN BRAINS........................................................ 171
R5- ATTEMPTS TO ESTABLISH HUMAN BRAIN
ENDOTHELIAL CELLS IN CULTURE........................................ 180
   A. Culture of astrocyte cell lines............................... 180
   B. Human umbilical cord endothelial cells.............. 186
   C. Growing human brain endothelial cells in culture. 188

INTRODUCTION OF THE T CELL PROLIFERATION
STUDIES................................................................................... 190

R6- T CELL PROLIFERATION STUDIES.......................... 191
   Abbreviations used in this section......................... 191
   Standardisation of the 'NTP T cell assay'................. 191
      a) Influenza virus antigens.................................. 192
      b) Purified protein derivative (PPD).................... 197
      c) Mycobacterium (M.) tuberculosis..................... 203
   The spotting technique........................................... 209
   The blotting technique........................................... 214
CONCLUSION OF THE STANDARDISATION OF THE T
CELL ASSAY ................................................................. 221

R7- THE T CELL PROLIFERATION ASSAY CLINICAL
STUDY..................................................................................... 222

R8- THE WESTERN BLOTTING STUDY............................ 227
   a) Autoantibodies against human brain vessels........ 227
   b) Autoantibodies against myelin......................... 230
DISCUSSION..................................................................................232
D1-THE CSF STUDIES.................................................................232
D2-CULTURE OF ENDOTHELIAL CELLS.................................235
D3-THE 'NITROCELLULOSE T CELL ASSAY'..............................240
   a/ Standardisation of the NTP T cell assay..........................241
   b/ The T cell assay clinical study...........................................244
D4-THE CLINICAL STUDY USING THE WESTERN
   BLOTTING TECHNIQUE..........................................................250
   a/ Discussion of the result of the western blotting assay
      with the purified vessels as antigens.................................252
   b/ Results with the myelin......................................................254
REFERENCES...............................................................................256
SUMMARY OF THE THESIS:

In this thesis, immunological parameters in multiple sclerosis patients (MS) have been examined and techniques contributing to the study of these aspects have been developed. The epidemiology of MS suggests that both environmental and genetic factors contribute to the disease process. According to a widespread theory, an autoimmune reaction leading to demyelination could be induced, in genetically determined individuals, by an infectious agent (s) e.g a paramyxovirus or a retrovirus.

In the first part of the thesis, the oligoclonal immunoglobulin (Igs) in the CSF of MS patients in relation to the paramyxovirus SV5 were studied. This was to complement an initial observation made in the department that a significant proportion of cerebrospinal fluids (CSF) could have oligoclonal bands directly acting against antigens (Ags) of this virus.

In the second part of the thesis, techniques were developed for the analysis of proliferative capacity of peripheral lymphocytes of MS and control patients using possible MS autoantigens viz myelin and brain vessels. The humoral response against these Ags was also assessed by immunoblotting using MS, neurological and normal control patients.

In parallel and in the view to obtaining antigens for the immunological studies, techniques for the purification and characterisation of human brain vessels from post mortem brains were assessed and attempts to culture human brain endothelial cell lines were made.

I: STUDIES ON THE SIGNIFICANCE OF THE PARAMYXOVIRUS SV5 IN MULTIPLE SCLEROSIS (MS).

This was tested in a series of 23 MS patients from Aberdeen, Dundee and Edinburgh. Particular attention was paid to the handling and storage of the CSFs which were screened initially by immuno-
fluorescence. Only eight of these CSFs showed any reaction against SV5/PF2 antigens. Two of these (with the highest titres) were then analysed for oligoclonal bands in the presence and absence of purified SV5 antigens and they failed to show any difference in the pattern obtained. In other experiments the effect of ionic strength, phosphate content and pH on the relatively cathodic oligoclonal IgGs suggested that some of the absorptions noted may have been due to non immune reactions.

In parallel with the immunoprecipitation technique, utilising standardised systems, the limits and use of other cerebral fluid characterisation techniques viz imprint immunofixation and immunoblotting technique were studied. The immunoblotting technique was extremely difficult to control and that it was prone to non specific reaction. On the other hand, the imprint immunofixation technique was easier to control but again the technique showed a narrow window of specificity.

In the light of these relatively negative results and in the light of current theories of the MS process it was decided to look at a related aspect of the aetiopathogenesis of MS which is the consideration of potential target autoantigens in the induction of MS.

**II. STUDY OF POSSIBLE AUTOANTIGENS IN MULTIPLE SCLEROSIS :**

It was thus decided to concentrate on 2 possible autoantigens: the myelin sheath and the brain vessels and to look at 2 arms, cellular and humoral, of the immune response (IR).

Concerning the cellular aspect of the IR, as very few antigens apart from myelin basic protein have been fully tested and because these types of studies have been hampered by the limitation in obtaining pure preparation of putative autoantigens, it was decided to use the 'nitrocellulose T cell assay' which has the potential to avoid difficult purification of protein. The nitrocellulose T cell technique utilises separation of antigens by SDS-PAGE followed by blotting onto nitrocellulose paper (NCP). The appropriate polypeptide antigen
band can then be cut out, dissolved in DMSO and reprecipitated as microparticles of antigen-NCP which can be used as antigen in a standard proliferation T cell assay. When the system was standardised and tested successfully with different antigens (adenovirus proteins, tetanus toxin and mycobacteria), NCP particles with myelin and human brain vessels (purified from post mortem brains) were prepared and tested with PBL from 6 MS patients and 6 neurological control patients from Dundee and Edinburgh and 6 normal controls from the department. Some reactivity was observed in 3 MS and 3 neurological controls against diverse myelin proteins and also against vessels proteins. However the level of reactivity was low (stimulation index between 1.5-3.5) but the batch of AB + serum used for the proliferation assay gave high background which may have masked a significant proliferation against a critical autoantigen. Nevertheless, in the context of autoimmunity and MS, this approach has been described and used successfully with peripheral blood mononuclear cells and thus its potential with T cell clones generated from the CSF and even also from the brain lesions is obvious.

A prospective blind study was also undertook with the sera from 45 MS, 47 neurological controls and 18 normal controls from the department using the 'western blotting' technique and myelin and brain vessels as antigens. There was no obvious difference in the pattern of reactivity between MS patients and other neurological controls. However there was a small proportion of individuals from both groups who possessed autoantibodies against either myelin or vessels which can be visualised by this technique. Among the normal control, only one reacted against a protein of the brain vessels (and not of the myelin).

III PURIFICATION AND CHARACTERISATION OF BRAIN VESSELS AND BRAIN ENDOTHELIAL CELLS.

Myelin purification is well described and its molecular composition and function have been well studied. Therefore effort was concentrated on the brain vessels where the purification technique is less well established.
There are a number of techniques which describe the purification of brain vessels from animal brains (rodent, canine and bovine) and from human post mortem brains. They are based on different principles which are often combined: 1/enzymatic digestion of the brain by 'collagenase, dispase', 2/mechanical homogenisation of the brain, 3/separation of the components of the brain by different gradient systems e.g dextran, sucrose 4/filtration through nylon sieves of different mesh sizes, 5/use of glass beads to trap the vessels. These different procedures have been assessed on the basis of yield and purity using bovine brains obtained from the local slaughterhouse and then on samples of human post mortem brains. The technique which was found to be the most adequate was a series of homogeneisations followed by filtrations through sieves of different mesh sizes (between 210 and 110 μ). The brain vessels are trapped on the sieve, collected and washed. The purity of the preparation is assessed by light microscopy and scanning electron microscopy. Characterisation of these purified brain vessels by known markers e.g Von Willebrand factor and gamma glutamyl transpeptidase was also undertaken.

Culture a brain endothelial cell line in vitro were sought and for this reason positive and negative markers of brain endothelial cell lines were collected and different culture conditions were tested. Two attempts using brain samples collected at temporal lobectomy from patients suffering intractable epilepsy were unsuccessful (although glial cell lines were grown for growth factors source).
INTRODUCTION.

BACKGROUND.

Multiple sclerosis (MS) is a neurological disease which affects the central nervous system; the lesions are characterised by a perivascular inflammation leading to demyelination - the destruction of myelin sheaths around the axons of neurons [Charcot J.M., 1868,a] [Adams C.W.M,1983]. MS affects mainly young adults (in their 3rd or 4th decades [Souberbielle B.E et al, 1990]) and as it is a chronic disease, generally marked by relapses and remissions, the cost to society and to the patient - socially and financially - is important. Moreover, there is still no treatment which has been shown to alter the long-term course of MS [McFarlin D.E., 1983]. The prevalence of MS is high in the north east of Scotland [Phadke J.G et al, 1987]; the highest prevalence of MS in the world has indeed been recorded in the Orkney islands and in the Aberdeen area [Editorials a) and b), 1981].

Although MS is not an hereditary disease, genetic factors play a major role in the disease process. The genetic predisposition seems to be polygenic [McFarlin D.E. et al,1989] [Oksenberg J.R. et al, 1990]. At least one disease gene is associated with the class II region of the HLA system on chromosome 6: associations have been described with the class II alleles DR 2 [Batchelor J.R. et al, 1978] and DQw1[Heard R.N.S. et al, 1989] [Vartdal F. et al, 1989]. Another disease gene seems to be linked to Gm allotypes on chromosome 14 [Pandey J.R. et al, 1981] and associations with both chains of the T cell receptor genes have been reported [Seboun E. et al, 1989][Oksenberg J.R. et al, 1989].

"MS epidemics", migrant and homozygotic twin studies indicate that a genetic factor is not the sole element in the aetiology of MS [Acheson E.D., 1985][Compston D.A.S., 1986]. It thus appears that one or even multiple external factor(s) are involved in the initiation and in the continuation of the disease [MacDonald, 1986]. The theory that an infectious agent(s) [Cook S.D. et al, 1980] induces demyelination in genetically determined individuals, by the mediation of an immunological reaction which could be autoimmune, is widespread [Waksman B.H. et al, 1984].
Many environmental agents have been implicated but infectious agents - particularly viruses - are on the top of the list and paramyxoviruses have been the focus of interest for a long time. Members of the department have pursued this line of research in London and then in St Andrews mainly on the SV5 virus - a member of the paramyxovirus family (see below)[Russell W.C.R., 1983] [Randall R.E.R. et al, 1989] and part of the work presented in this thesis is the continuation of this investigation.

Another important line of research in MS is based on the hypothesis that MS is due to an autoimmune attack [Waksman B.H. et al, 1984] and, as already mentioned, this hypothesis could be compatible with a possible viral etiology of MS. As MS is a demyelinating disease, myelin has been investigated as a possible target by many research groups in the world [Hafler D.A et al, 1989] especially the myelin basic protein which has been shown to induce experimental allergic encephalomyelitis (EAE) in animals [Ben-Nun A et al, 1981]. However, MS researchers are now also focussing their attention on the blood brain barrier (BBB), the microvessels which supply blood and nutrition to the brain and on the possibility that it may be the primary site of the MS process as the lesions of MS are strikingly perivascular (see below) [Adams C.W. M., 1989]. The second part of this thesis presents data related to both areas (autoimmunity and BBB).

MS is undoubtedly a very complex disorder. It is also a difficult disease to work on, mainly because of the inaccessibility of the site of the lesions (the central nervous system) in live patients. Despite this limitation, a mass of information about many aspects of MS has been collected during more than a century of research since the first descriptions of this frustrating but nevertheless fascinating disease [Charcot J.M., 1868,b][Critical digest, 1896][Compston D.A.S., 1988]. In order to clarify the aim of this thesis, brief reviews on the clinical symptoms of MS, the description of the MS lesions, the immunology of MS, the cerebrospinal fluid (CSF) in MS, the association of viruses with MS and the BBB and MS will follow in addition to a brief review of the immunological techniques used in this thesis.
CLINICAL ASPECTS OF MS.

MS is characterised clinically by episodes of isolated neurological symptoms (spinal cord, optic nerve and brain). Although pleomorphic in its clinical presentation, the picture seems to be determined by the location of the MS lesions. Classic features include impaired vision, nystagmus, dysarthria, intention tremor, vertigo, ataxia, impaired position and vibratory senses, bladder dysfunction, weakness or tingling of a limb and alteration in emotional responses [Poskanzer D.C. et al, 1981].

The course of the disease differs between individuals and there are no known prognostic factors. Broadly, in about 15% of cases MS is completely asymptptomatically silent and only found at autopsy [MacKay R.P. et al, 1967] [Morariu M. et al, 1976]; in 15% the course of MS is 'benign' (it does not interfere with the social life of the patients); in 55% of cases patients are disabled by MS and in another 15% of cases patients MS is the cause of death. Among the patients who show signs of the disease about 90% have a relapsing-remitting form (Charcot's type) and 10% a progressive disease (the latter patients are usually older) [Compston D.A.S., Conference on Positive trends in multiple sclerosis research, Royal College of Physicians, 1991]. Optic neuritis is a frequent initial symptom (about 40% of the cases) and about 60-75% of patients who have had an episode of optic neuritis will go on to develop other lesions of MS [Francis D.A. et al, 1987]. It is thus unclear if an isolated episode of optic neuritis can be considered as a subclass of MS or a different disease [Kinnunen E. et al, 1989]. Other rare clinical subsets of MS have been described and named e.g Neuromyelitis Optica (Devic's form) but as they do not correlate with a specific prognosis they are of limited value. One uncommon type worth mentioning is the acute form of the disease or Marburg's type where MS runs an acute or subacute course leading to death in weeks or months. The differential diagnosis in this situation is disseminated encephalomyelitis.

It is not known what causes the symptoms in MS. Recent nuclear magnetic resonance (NMR) studies in patients suffering from MS have shown that multiple lesions can appear and disappear without any clinical consequences whereas other lesions, at the same time, can give rise to a clinical attack [Jacobs L., 1986] [Ormerod
I.E.C., 1986]. The location of the lesion is likely to be a major factor in the appearance of clinical symptoms but it is thought that the functional defect is due to the oedema around the inflammatory lesions and not due to the cellular reaction in the lesion. It is also now thought that chronic disability may be due to the axonal loss in old lesions [McDonald W.I, Conference on Positive trends in multiple sclerosis research, Royal College of Physicians , 1991]: new lesions are characterised by the sparing of axons but older lesions are marked by scarring due to gliosis which lead also to axonal loss (see below [Adams C.W.M., 1983]).

Because of its nature, history and clinical diversity, it is evident that MS is a difficult disease to diagnose and to assess in terms of progression. Moreover, a diagnosis of multiple sclerosis raises the uncertainty of disability and loss of independence in a patient. Although NMR, when available, greatly helps in making a diagnosis of MS, there is still no specific diagnostic test and thus the diagnosis remains clinical for most doctors. 'Definite MS' is characterised clinically by the presence of at least 2 separate lesions that have appeared at different times in the white matter of the central nervous system [McDonald W.I., 1989]. Other possible causes of neurological dysfunction need to be excluded e.g HTLV-1 associated neuropathies [Tournier-Lasserve E. et al, 1987]. The diagnosis is much more difficult in a patient with a progressive, non-remitting pattern [McDonald W.I., 1989].

It is essential that clinical guidelines are being followed and can be compared when clinical researchers select patients suffering from MS for scientific projects or for therapeutic trials. It is also important to restrict therapeutic trials and other research protocols to 'definite MS' cases. Nowadays, the accepted criteria for diagnosis are those of Poser et al [Poser C.M. et al, 1983] and one needs to emphasize that the diagnosis must only be made by a neurologist. Disability status scales have also been published [Kurtzke J.F., 1970].
THE HISTOPATHOLOGY OF MS.

Describing the MS lesions, understanding their evolution and their histories and comparing their locations are crucial scientific activities which give an overall chronological picture of the MS process and which may guide MS researchers on the trail of the etiological factors of MS. They may also identify elements or stages of the MS lesions which could be targets for successful therapies even though these treatments are not directed to the cause of MS [Compston D.A.S., 1990]. As pointed out before, this scientific approach is hampered by the inaccessibility of the MS lesions in live patients. Post mortem tissue is the only source of lesions for histopathological studies and the MS lesions at post mortem are often old. As a brain biopsy on a live MS patient may be harmful, it is unethical to biopsy a patient for research purposes only: fresh demyelinating lesions have been reported near the needle tract after stereotactic destruction procedures for severe tremor in MS patients [Gonsette R. et al, 1966] [Riechert T. et al, 1975].

Location of the MS plaques.

MS lesions, or plaques, commonly involve the central white matter but some pathologists have reported their presence—sometimes in greater number—in the grey matter [Adams C.W.M., 1983 and 1989] [Lumsden C.E., 1970]. Macroscopically, they measure between 1 to 40 mm and are pinkish when they are new whereas older lesions are grey [McFarlin D.E. et al, 1982]. MS lesions favour particular locations which are seen at post mortem but also with great accuracy by NMR: they are extremely frequent (in over 90 % of the cases) in the periventricular regions and around the aqueduct (especially near the lateral and the 4th ventricles and less commonly around the 3rd ventricle) [Adams C.W.M., 1983] [McDonald W.I., 1989]. They are also commonly found in the optic tracts and frequently in the cord, pons, mid brain and basal ganglia. Plaques are twice as common in the cervical area of the spinal cord as in the lower part of the cord [Oppenheimer D., 1978][Poser C.M., 1986].

In 20 % of MS patients, a perivenular exudate is present around the retinal veins and this phenomenon is called periphlebitis retinae (see figure 4 in [Bates D., 1986]). Periphlebitis retinae seem

Lymphocytic meningitis is another feature of the MS disorder which is little understood. It may manifest itself as perivenular meningeal infiltrate or as a more diffuse meningeal inflammation. It is associated with acute cases of MS although it is not present in all acute cases. The lymphocytes are predominantly located in the subarachnoid space, particularly in the depths of the sulci (the space between the 2 cerebral hemispheres) [Adams C.W.M., 1977].

Perivascular situation of the MS plaques.

Microscopically, it has been recognised, since the first histopathological descriptions of MS lesions [Charcot J.M., 1868,a] [Critical digest, 1896], that the characteristic MS plaques are located around brain vessels and almost exclusively around venules and small veins [Adams C.W.M., 1983 and 1989] and particularly in acute cases of MS [Adams C.C.W. et al, 1989, b]. It has been shown by serial studies that some plaques can extend over a considerable distance along the course of a vein [Fog T., 1964 and 1965] and this is particularly common in the spinal cord [Lumsden C.E., 1970]. The lesions can also spread out along the course of a vein as 'Dawson's fingers'. These latter lesions often occur in the periventricular regions (the periventricular lesions are thus related to the periventricular veins and not to the ventricular lining or ependyma) [Fog T.,1965] [Adams C.W.M, 1987]. With NMR it has been shown that the MS lesion seems to develop in a centrifugal fashion [Kermode A.G. et al, 1990, b]. In some cases, MS lesions are thought to be composed of multiple smaller lesions [Adams C.W.M., 1989].
Relapsing-remitting MS and primary progressive MS may be 2 different diseases.

Recent NMR findings have shown that the pathogenesis of primary progressive multiple sclerosis appears to be different from the relapsing-remitting form. Gadolinium-DTPA has been used to assess the permeability of the BBB in conjunction with the NMR technique: it is a liquid of contrast which does not normally cross the BBB but in MS when the BBB is disrupted, it is able to leak into the brain and the lesions show enhancement by NMR. The MS lesions in early stages of primary progressing MS do not 'enhance' with Gadolinium-DTPA whereas they enhance in relapsing-remitting form of the disease [Thompson A.J. et al, 1989].

Histopathology of the 'active MS plaque'.

a) The different cell types in the MS plaque - some general considerations.

The brain is composed of different cell components (the brain cells themselves and the other cells). The actual brain cells are the neurons and the neuroglial cells (astrocytes, oligodendrocytes, microglial cells and ependymal cells); they all are of neuroectodermal origin apart from the microglial cells. Microglial cells, of mesodermal origin, are endogenous phagocytic brain cells which are thought to have migrated (as macrophages?) from the cerebral circulation into the nervous system in response to neuronal death early during development and which do not express markers of haematogenous macrophages [Esiri M.M. et al, 1986] [Esiri M.M. et al, 1988]. Microglial cells will thus be considered with the macrophage-monocyte cells. Ependymal cells are the cells lining the ventricles of the brain and do not seem to be involved in the MS process.

Other cells which make-up the brain are the meningeal cells, the cells which form the brain blood vessels and the connective tissue cells and are all of mesodermal origin. They will be dealt with in the relevant sections.

The cellular infiltrate - mainly lymphocytic - which characterises the MS lesions is thought to be principally of
haematogenous origin and will be discussed separately. The blood vessels and endothelial cells will be discussed in the blood-brain barrier section.

b) Histopathological features of the MS lesion.
A schematic representation of a MS plaque is shown in fig. HISTO-1 at the end of the histopathology section.

In an 'active MS plaque' (relapsing-remitting form), there is a peripheral zone of active demyelination where the myelin is only partially degraded. These peripheral zones show some hypercellularity which is spread throughout the plaque in active lesions. The hypercellularity appears to be due to the response of brain cells (astrocytes, microglial cells and oligodendrocytes) to the MS insult: this has to be differentiated from the inflammatory infiltration. Sudanophilic or osmophilic myelin lipid breakdown products are visualised within the macrophages or the microglial cells which are actively taking up and digesting the myelin [Adams C.W.M., 1989].

Within the plaque there is an increase of proteolytic enzymes and a loss of lipids and proteins (MBP and myelin associated glycoprotein (MAG)) which make up the myelin [Adams C.W.M., 1989]. Interestingly, MAG seems to be lost earlier and to a greater extent than other myelin proteins at the edge of the MS lesions [Itoyama Y. et al,1980][Moller J.R., 1987].

Other criteria of activity are the presence of oedema and fibrinous exudation [Adams C.W.M. et al, 1985] as well as IgG and complement deposition. Oedema is frequent and is probably the first event in the formation of a MS lesion [Kermode A.G. et al, 1990 a and b]. Fibrinous exudation and the other pathological features are however not always present in 'active MS plaques' [Adams C.W.M., 1989]. Complement deposition (C9) appears to be restricted to the brain vessels on the lumen but predominantly on the parenchymal side [Compston D.A.S. et al, 1989]. C3 shows a more diffuse perivenular distribution [Adams C.M.W. et al, 1985]. Complement has also been demonstrated in neuroglia, at the edge of active plaques and in the subependymal region of MS brains [Esiri M.M., 1977 and 1980] [Adams C.W.M. et al, 1987]. IgG deposition has been detected in plaques, in the perivenular infiltrate and is also visible in astrocytes and microglial cells at the edge of the plaques [Prineas J.

All these features are the mark of an inflammatory reaction and could be associated with increased permeability of the blood-brain barrier within the 'active region'. However in the case of IgG production and complement activation, it has been shown that they also happen intrathecally [Walsh M.J. et al, 1983][Compston D.A.S. et al, 1989].

c) The oligodendrocytes in the MS plaque.

Oligodendrocytes are the cells which produce and maintain the myelin in the central nervous system. The myelin sheaths which originate from a single oligodendrocyte enfold short sections (30 to 50) of many neighbouring axons within the CNS [Peters A. et al, 1970]. This contrasts with the situation in the peripheral nervous system (PNS) where the Schwann cell (the myelin forming cell of the PNS) is associated with one axon and produces only a single myelin segment [Gregson N.A., 1983]. In the CNS, between the axonal portions enveloped by the oligodendrocyte processes, bare axonal segments are found and are named nodes of Ranvier. The nodes of Ranvier are covered by the foot processes of type 2 astrocytes [Compston D.A.S., 1990]. Type 2 astrocytes and oligodendrocytes are derived from a common precursor cell, the O-2A progenitor cell [Raff M.C. et al, 1983]. Markers for oligodendrocytes can be detected by antibodies against galactolipid, carbonic anhydrase and transferrin [Adams C.W.M, 1989].

Two contradictory phenomena have been observed in the 'active MS' plaques. In some instances, the number of oligodendrocytes is very much decreased in the plaques and demyelination may thus result from this loss of myelin-producing cells. However it has also been observed that oligodendrocytes persist and even proliferate at the demyelinating edge of the plaque [Raine C.S. et al, 1981]. This phenomenon may be due to recruitment of new oligodendrocytes by the glial progenitor cells but also it may be the consequence of a non specific response to the inflammatory reaction. For instance it has been shown that interleukin 2 - the main cytokine secreted by activated lymphocytes - stimulates oligodendrocyte proliferation [Benveniste E.N. et al, 1986].
It is still disputed whether the oligodendrocyte loss in MS is primary or secondary. The hypothesis that the oligodendrocytes may be injured in MS by auto-antibodies [Abrawsky O. et al, 1977] has not been confirmed [Traugot U. et al, 1979].

More recently Compston and his colleagues revived the interest in oligodendrocytes and MS as they showed with others that oligodendrocytes are particularly susceptible to the action of complement [Compston D.A.S., 1990]. It appears that activation of the classical pathway, in the absence of antibody, is sufficient to induce oligodendrocyte cytotoxicity [Wren D.R. et al, 1989] and this mechanism is enhanced in the presence of any anti-oligodendrocyte antibodies and macrophages [Scolding N.J. et al, 1989]. However, there is no consensus about the presence of complement on oligodendrocytes in the MS plaques and this may be due to the difficulty in detecting complement components with immunocytochemical techniques [Gay D., Conference on Positive trends in multiple sclerosis research, Royal College of Physicians, 1991].

d) The astrocytes in the MS plaques.

The astrocytes are the supporting framework for nerve and blood vessels in the brain. They are located throughout the CNS and are particularly concentrated immediately under the pia matter and under the ependyma. They act as transport channels for the conduction of water and electrolytes within the CNS. Their many cytoplasmic processes make contact with the node of Ranvier of the axons and with the brain blood vessels. Most of the brain specific properties of the brain vessels are induced and maintained by the astrocytes [Golstein G.W., 1988] [Janker R.C. et al, 1987]. They also produce and secrete soluble factors which influence the brain cells and the supporting cells. The astrocytes have been shown to possess phagocytic properties and can act in vitro as antigen presenting cells [Fontana A. et al, 1984]. Astrocyte markers are glial fibrillary acidic protein (GFAP) and others less specific like vimentin [Lagenaur C.F., 1984].

Astrocyte activation and proliferation is a common feature of the active MS plaques and it seems that it is the first CNS cell to display overt reactivity during the development of MS lesions. Astrocytes show overt reactivity well into the adjacent unaffected
parenchyma [Prineas J.W. et al, 1976]. Astroglial hypertrophy is not specific for MS as it is found in other conditions like viral infection, stroke, trauma and tumours [Raine C.S., 1988]. Immunoglobulins phagocytosed by the astrocytes in MS plaques is a common feature [Prineas J.W. et al, 1976]. Astrocytes are also known to participate in myelin phagocytosis [Raine C.S., 1988]. It is still a matter of contention whether astrocytes in the active MS lesions are class II MHC positive [Traugott U. et al, 1985] or not [Hayashi T., 1988]. In summary, it is still unclear if astrocytes have a primary role in the genesis of the lesions e.g as phagocyte cells or APC or if their behaviour seen in the MS plaques is only a secondary response to the insult generated by other factors.

The inflammatory cellular infiltrate in the active MS plaque.

The cellular inflammatory reaction is characterised by a perivenular lymphocytic infiltration and is composed mainly of T lymphocytes (CD4 and CD8 positive cells) but also of monocytes, macrophages, B cells and plasma cells. It ranges from a modest to a more severe infiltrate as seen in vasculitis e.g in polyarteritis nodosa (in acute viral disease such as polyomyelitis the cellular infiltrate may be quite modest) [Adams C.W.M., 1989]. Adams and colleagues have observed that in some MS lesions the lymphocytic infiltration was initially confined to the cerebral vessel wall alone and this may be a feature of very early MS lesions [Adams C.W.M. et al, 1985]. Lymphocytes then seem to accumulate in the Virchow-Robin space between the vessels and the brain interstitial side. The point of maximum immigration of lymphocytes into the perivascular space seems to be the venocapillary junction [Adams C.W.M., 1989].

The cellular infiltrate has been described in detail by many researchers but it is not always clear from the literature which patient groups were used as source of the analysed brain material (remitting-relapsing or progressive MS). A summary of these findings follows.
a) The T cell component of the cellular infiltrate.

There is debate about the proportion of the T cell subsets (CD4+ versus CD8+ cells) in the 'active MS lesions'. Immunocytochemical studies on fresh MS plaques, on balance, have revealed that suppressor/cytotoxic T cells predominate over T helper cells [Boos J. et al, 1983] [Woodroffe M.N. et al, 1986] [Hauser S.L. et al, 1986] [Hofman F.M. et al, 1986] [McCallum K. et al, 1987] [Hayashi T. et al, 1988]. When the proportion of the T cell subsets was correlated with the appearance of activity of the different plaques, it appeared that the active phase of a lesion was characterised by an increased number of T helper cells and therefore it was concluded that T helper/inducer cells are necessary for plaque expansion. On the other hand, plaques which were static showed an increased number of CD8+ cells and therefore T cytotoxic/suppressor cells were thought to be associated with controlled lesions [McCallum K. et al, 1987] [Raine C.S. et al, 1988]. On the contrary, Woodroffe et al found that CD8+ cells were associated with widespread staining of class I MHC antigen in the MS lesion (a prerequisite for antigen presentation to class I restricted cytotoxic T cells) and thus concluded that these CD8+ cells may function as cytotoxic instead of suppressor cells [Woodroffe M.N. et al, 1986].

In the same way there is much debate on the proportion of activated T cells (e.g. IL2 receptor positive cells) within the 'active plaque': Woodroffe and colleague have observed a high proportion of activated T cells (10 to 40% of total cells) [Woodroffe M.N. et al, 1986] whereas Hayashi and colleagues found very few activated T cells in the MS plaques [Hayashi T. et al, 1988]. Secondly, it is not clear what is the proportion of T cells at the edge of the lesion and how many T cells can be seen in the outside proximity of the MS lesions: overall, T cells at the periphery are usually scanty and always outnumbered by those in the perivascular space but nevertheless they seem to peak at the plaque edge in comparison with the rest of the plaque [Adams C.W.M., 1989][McCallum K. et al, 1987].
b) The B cell component of the cellular infiltrate.

It appears that the T cells precedes the B cell in the inflammatory reaction [Raine C.S. et al, 1988]. Although B cells are not represented at the hypercellular plaque borders, they sometimes account for a high proportion of the cells in the cellular infiltrate of the perivascular cuff (up to 40% of the total cells) [Woodroofe M.N. et al, 1986]. It has also been suggested that B cells are 'trapped' in the MS lesions and produce oligoclonal bands. Plasma cells are also seen in the cellular infiltrate and, in some cases, their proportion may be high (up to 10%) and may be a feature of more chronic lesions [Prineas J.W. et al, 1978]

c) The monocyte-macrophage component of the cellular infiltrate.

Macrophages/microglial cells are part of the hypercellularity seen in active MS lesions. Again there is no real consensus about the role played by the macrophages in the initiation of the MS lesions and their role in demyelination. Some have suggested that haematogenous macrophages (e.g acid esterase and phosphatase positive) enter the lesions early and constitute most of the 'foam cells' (the cells which actively phagocytose the myelin). They then lose some of their markers and become resident macrophages [Esiri M.M. et al, 1988]. In older lesions the foam cells lack most of the markers specific for haematogenous macrophages and thus it is not clear if these cells originate from the cerebral vasculature and then lose their specific markers or if they are mainly endogenous microglial cells.

While scattered lipid-laden microglia/macrophages can be found throughout the white matter and plaques in MS, in 'active plaques' they populate the areas of intense demyelination where they seem to 'pick up' (sometimes seen as 'myelin stripping') the myelin in the form of globules (myelinic bodies). In some cases they are seen in contact with the demyelinating edge of the plaque [Raine C.S. et al, 1988][Adams C.M.W., 1989] and capping of IgG has been seen in macrophages in contact with the myelin [Prineas J.W. et al, 1981]. Nevertheless, it is still not clear if macrophages/microglial cells are primarily responsible for the demyelination or if they only exert a secondary scavenger response to the myelin destruction.
One feature which favours a direct role in demyelination is the immunoglobulin-capping of macrophages which has been described in some active lesions [Prineas J.W et al, 1981]. Macrophages and microglial cells could also act as antigen presenting cells: they have been shown to express a large amount of class II but also a small amount of class I MHC molecules in MS lesions [Hayashi T. et al, 1988] [Woodroffe M.N. et al, 1986].

**The chronic MS lesion.**

The plaque may continue to be active at its periphery with a modest lymphocytic infiltrate or occasional foam cells. The chronic lesion shows an abundance of astrocytes and spider cells which accounts for most of the scarring in 'old plaques'. In such cases, axon loss may be seen; this Wallerian degeneration is unidirectional as it spreads along the length of the neuron in contrast to the spread of the MS lesion which is centrifugal.
Figure HISTO-1: Schematic representation of a MS plaque.
THE BRAIN VESSELS IN MS.

General considerations: the BBB.

a) Definition and function of the BBB.

The demonstration by Paul Ehrlich that intravenously injected vital dyes did not stain the brain and the spinal cord but stained other organs, has given rise to the concept of a barrier between the blood and the brain [Ehrlich P., 1885]. The BBB is present in virtually all vertebrates but absent in the brains of invertebrates. Thus in evolutionary terms, the BBB parallels the myelin as myelin is lacking in the invertebrate nervous system [Pardridge W.M. et al, 1986, b]. The main morphological feature of the BBB is the presence of high-resistance tight junctions (or occluding junctions) which seal the brain endothelial cells together into a continuous tube separating the blood from the brain tissue. The molecular description of these tight junctions of the BBB is virtually still lacking but it appears that the properties of the BBB are induced and maintained by the local environment and especially by the astrocytes [Stewart P.A. et al, 1981] [Janzer R.C. et al, 1987] [Golstein G.W., 1988] [Pardridge W.M. et al, 1986].

In the human healthy brain, the BBB is omnipresent except for about half a dozen of small areas in the circumventricular organs such as the median eminence of the floor of the third ventricle, the area postrema surrounding the caudal tip of the fourth ventricle, and the choroid plexus: these regions are probably sites of local exchange between the blood and brain for peptides or other substances which may then have access to the cerebrospinal fluid (CSF) through the low-resistance tight junctions of the ependymal cells lining the surface of the ventricles. The BBB therefore is to be differentiated from the blood-CSF barrier in terms of structure and in terms of function. Moreover, the composition of the brain interstitial fluid and of the CSF are different.

The transport through the BBB is selective and regulates the entry of fluid, electrolytes, small molecules and proteins. It maintains the composition of the brain extracellular fluid which is very different from that of the blood plasma especially by keeping a low concentration of proteins in the brain interstitial fluid. The
transport through the BBB is governed mainly by the specific chemical characteristics of the penetrating substance rather than by its molecular size. On the BBB, specific membrane proteins (carrier proteins) involved in the transport of specific molecules have been described e.g for insulin [Pardridge W.M. et al, 1985, b] or for phenylalanine [Choi T.B. et al, 1986]. Conversely, the BBB is known to be highly impermeable to the centrally active neurotransmitters and thus prevents their loss to the blood plasma after their synthesis in the brain. However, the BBB is not totally impermeable to the plasma proteins as a minimal degree of leakage can be observed: this phenomenon may be due to a residual pinocytosis seen in the brain vessels although pinocytic vesicles are characteristically minimal in the brain endothelial cells [Pardridge W.M. et al, 1986, b] [Reese T.S et al, 1967].

b) Anatomical structure of the BBB.

For a complete understanding of the BBB, one first has to understand the organisation of the vascular tree. The blood is confined to a close circuit of vessels lined by a simple squamous epithelium, the endothelium. For the entire vasculature, an approximate number of $6 \times 10^{13}$ endothelial cells is estimated and their solid mass should approximate 1 kg. It is now known that endothelial cells differ in their nature and their composition between different organs and also between different types of vessels within the same organ [Zetter B.R., 1981 and 1988] [Rupnick M.A. et al, 1987].

The vascular tree is made up of different types of vessels organised in series from the arterial to the venous system as follows: elastic arteries, muscular arteries, arterioles, capillaries, pericytic venules, muscular venules and small, medium and large-sized veins. Vessels larger than 100 μm in diameter are considered part of the macrovasculature whereas smaller vessel are part of the microvasculature. The microvasculature corresponds to the arterioles, capillaries and emerging venules as well as the arteriovenous anastomoses. Anatomically speaking, the BBB involves only the microvasculature and more specifically the capillaries and their emerging venules as it is only in these types of vessels that exchanges between blood and tissue occur. The vessels in the rest of the vasculature tree possess a continuous vessel wall which prevents significant exchanges [Simionescu N. et al, 1990].
Continuous capillaries are not only a feature of the CNS as they are also found in muscular tissue (skeletal, cardiac and smooth muscle) but also in the exocrine pancreas and the gonads [Palade G.E., 1961] [Simionescu N. et al, 1990]. The structure of the vessels is organised into three layers or 'tunics': the intima, the media and the adventitia. In the brain capillaries, the intima consists of a single layer of thin endothelial cells and a basal lamina (basement membrane) in close contact with the pericytes. The endothelial cells are joined together by tight junctions. These occluding junctions display a different organisation from that seen in other epithelia [Simionescu N. et al, 1990]. The media is normally composed of muscular cells but it is virtually lacking in the brain capillaries. The adventitia consists of the brain connective and neural tissue which, in the brain, is tightly packed around the vessels. The passage of substances from the blood to the brain is further restrained by the foot processes of the astrocytes which are closely applied to the vessel wall and cover 70% of its surface [Golstein G.W., 1988].

The postcapillary (pericytic) venules range from 10 to 50 μm in diameter and appear to have a comparable vessel wall as the transition between the capillaries and the venules occurs gradually. Immunologically, the post capillary venule is an important site for inflammation and allergic reactions and exhibits a high concentration of adhesion molecules [Raine C.S. et al, 1990]. Prineas has also described structures resembling lymphatics around the brain vessel especially in the Virchow-Robin space [Prineas J.W., 1979]. The Virchow-Robin space (VRS) has recently been reviewed by Esiri and Gay [Esiri M.M. et al, 1990]. The VRS is the space between the brain vessels and the cerebral matter. The VRS is now known to be an extension of the subpial space rather than of the subarachnoid space in man. The pia is made of a single layer of cells linked together by desmosome and gap junction. From the pia, the space extends as far as the capillaries. At this level the VRS is occluded by the glia limitans formed by the foot processes of the astrocytes.
Endothelial cells (EC) and brain endothelial cells.

a) The endothelial cells and their characterisation.

Information on the biology of the EC has been amassed during the past decades because of the important role of these cells in the biology of the human body: they cover the lumen of blood vessels and lymphatics, they have an important role in homeostasis and in the permeability of blood vessels and mediate response to physiological and pathological stimuli by excreting soluble factors and expressing new molecules [Vane J.R. et al, 1990] [Dinh Xuan A.T. et al, 1989]. It is nevertheless important to stress that the source of EC for most of these studies was the macrovasculature namely the vein of the umbilical cord because of the accessibility of this organ in live patients and because of the relative ease in culturing human umbilical vein endothelial cells (HUVEC) [Jaffe E. A., 1977]. It is mainly because of Folkman and his colleagues in the late seventies that culture of EC from the microvasculature became feasible [Folkman J. et al, 1979]. However, EC from the microvasculature of some organs are still very difficult to propagate in culture: there is no universal method as it appears that the optimal conditions of culture for these cells are different as a function of the organ of origin [Pearson J., 1990].

Typically, EC in culture are flat, thin, of cobblestone morphology with large round nuclei and are contact inhibited whereas cultured smooth-muscle cells and fibroblasts grow as overlapping spindle-shaped cells [Jaffe E.A., 1977]. Some markers specific for EC are now available. The best known is von Willebrand factor (vWf) protein which is synthetised by EC and megakaryocytes and is found at low concentration in the plasma [Wagner D.D. et al, 1982]. The confusion between vWf protein and factor VIII - in some cases, described as a specific marker for EC - arose as it was not possible to measure these 2 compounds separately as vWf was part of the so called 'factor VIII related antigen' [Jaffe E.A., 1977]. However factor VIII is not synthetised in EC but in the liver and not found in EC but in the plasma. Other less established and also less specific markers are used and can be measured by immunohistochemistry, when there is a monoclonal antibody available, or, by a biochemical assay. For example angiotensin-
converting enzyme (A.C.E) is considered as indicative of EC [Caldwell P.R.B. et al, 1976] [Auerbach R. et al, 1982] even if it is also found in alveolar macrophages, in certain epithelia, in brush cells of the gut, and in the proximal tubes of the kidneys [Zetter B.R., 1981]. ACE activity often decreases in culture [Del Vecchio P.J. et al, 1981] as does vWF expression [Gerhart D.Z. et al, 1988]. A number of lectins have been shown to be specific for the EC; the best known being Ulex Europaeus Agglutinin I (UEA-I) in humans [Holthofer H. et al, 1982]. However, these lectins do not seem to be universal in all species e.g UEA-I does not bind canine EC whereas Ricinus communis agglutinin I (RCA-I) does [Gerhart D.Z. et al, 1986]. EC express other molecules like the antigens recognised by the monoclonal antibodies PAL-E and EN4 which are also endothelial cell specific [Schlingemann R.O. et al, 1985 and 1991]. They (HUVEC) also express many immunological markers e.g ICAM I (CD54), CD29, CD34, CD 31, GP IIIa, and OKM 5 [Romano P. et al, 1989]. The antigens corresponding to CD 31 (ENDO-CAM) is considered to be an endothelial marker but is also present on platelet as platelet GP IIa antigen and is also present on a number of other blood cells [Albeldo S.M. et al, 1990]. CD 34 has been shown to be expressed in dividing endothelial cells during angiogenesis, therefore expressed in the vasculature of a number of tumours [Schlingemann R.O. et al, 1990].

Other assays for the characterisation of EC have been described e.g the 'platelet adhesion test' that ascertains that EC have a nonthrombogenic surface [Wechezak H.R. et al, 1979] or the demonstration of the uptake and degradation of LDL by endothelial cells by the presence of acetylated low density lipoprotein [Voyta J.C. et al, 1984] although the latter test doesn't seem to be positive with EC from all origins [Rupnick M.A. et al, 1988]. By electromicroscopy, the specific endothelial organelles or Weibel-Palade bodies can be seen in human EC from the macro- and microvasculature [Wagner D.D. et al, 1982] [Folkman J. et al, 1979]; they have been shown to contain vWF protein [Wagner D.D. et al, 1982].
b) The brain endothelial cells.

EC from the brain microvasculature of some animals (mouse, cow, guinea-pig) have been maintained in culture by some groups [Bowman P.D. et al, 1983] [Audus K.L. et al, 1986] [Wilcox C. et al, 1989]. However, reports of human brain EC culture have been rare [Gerhart D.Z. et al, 1987] [Vinters H.V. et al, 1987] and biological and molecular characterisation of these cells - other than their morphology and the description of expression of some markers - is still lacking.

Brain EC from all species express the specific endothelial markers (vWF, ACE) and sometimes show some brain specific properties. However, the main difficulty in the characterisation of brain EC in culture is that they appear to lose these brain and endothelial specificities rather quickly.

Brain specific characteristics of EC are likely to be related to the function of the BBB. For example gamma-glutamyl transpeptidase (γ GTP) has been used as a marker for brain- and retinal-derived EC [Pardridge W.M. et al, 1986]. The enzyme is important in glutathione metabolism and amino acid transport: it catalyses transfer of the γ - glutamyl residue of glutathione to amino acids. It is not limited to cerebral endothelium but seems to be present only in cells with active transport properties and polarity transport, such as biliary epithelia and renal tubular epithelial cells [Orlowski M. et al, 1974] [DeBault L.E. et al, 1980]. Another enzyme which is claimed to be specific for brain EC is alkaline phosphatase [Audus K.L. et al, 1986] but this enzyme is also present in other cells e.g HELA cells a cancer derived cell line from the cervix [W.C Russell, personal communication]. Monoclonal and polyclonal sera have been produced and seem to be specific for the brain EC but these sera are not universally available: N. Sternberger and colleagues made a monoclonal antibody called anti-EBA which reacts with rat brain EC and is specific for a protein triplet of 23,500, 25,000 and 30,000 Kd [Sternberger N.H. et al, 1987] (this monoclonal antibody is now commercially available). A polyclonal sera specific for a 46 Kd protein seems to be also specific for the BBB [Pardridge W.M. et al, 1986]. On the other hand, the antigen recognised by the monoclonal antibody Pal-E is absent from endothelium in sites with an intact BBB but is present in some brain
tumors where the BBB is likely to be disrupted [Leenstra S. et al, 1990].

With electronmicroscopy, some have demonstrated the presence of tight junctions between brain EC in culture and freeze fracture revealed a complex anastomosing network of intermembranous ridges [Bowman P.D., 1981]. Brain EC have characteristically very few pinocytic vesicles contrary to other sources of EC. They do not appear to contain Weibel-Palade bodies which are thought to be mainly a feature of the macrovasculature although Weibel-Palade bodies have been observed in EC from the microvasculature [Weibel E.R. et al, 1964] [Folkman J. et al, 1979].

Most of the immunological features demonstrated by animal brain EC have also been demonstrated with HUVEC or other types of EC (for a review see [Pober J.S., 1988]). Although normal brain EC do not express class II MHC molecules, the expression of this molecule is inducible by INF-γ as is the expression of class I MHC [Male D.K. et al 1987]. By immunohistochemistry it has been shown that brain EC also express a number of adhesion molecules [Raine C.S. et al, 1990]. Both features are likely to be important in leukocytes and especially lymphocyte interactions. For example, adhesion of lymphocytes to cerebral EC is increased by INF-γ, TNF and IL 1 [Hughes C.C. et al, 1988]. Moreover, both MHC and adhesion molecules are critical for antigen presenting capacity which has been demonstrated in rodent brain EC [Wilcox C.E. et al, 1989].

The brain pericyte and smooth muscle cell.

The vascular smooth muscle cell is the main cell of the subendothelial connective tissue in all vessels, except, in capillaries and pericytic venules, where this place is taken by the pericyte which is probably a variant of a smooth muscle cell.

Some groups have described the culture of smooth muscle cells from mice brain microvessels: these cells actively produced α-actin, a smooth-muscle-specific isoactin. However, the differentiation between brain smooth muscle cells and pericytes is not straightforward [Moore S.A. et al, 1984] [Larson D.M. et al, 1987]. Nevertheless, they have shown that these smooth muscle cells can express class II MHC antigens in vitro [Hart M.N. et al, 1987] and
that this MHC expression is inducible by INF-γ [Fabry Z. et al, 1990]. These cells have been shown to act as antigen presenting cells and this antigen-presenting capacity is also enhanced by INF-γ [Fabry Z, et al, 1990].

The immunological interest in smooth muscle cells is further bolstered by the demonstration of autoimmune vasculitis resulting from sensitisation of lymphocytes to smooth muscle cells [Hart M.N. et al, 1985].

**Historical background of the brain vessels in relation to MS.**

The importance of the brain vessels in the aetiopathogenesis of MS was recognised soon after the first clinical descriptions of MS. For example, the conclusion drawn from one of the first international symposia on MS almost a century ago was: "... These changes are very suggestive that the disease is associated with vascular lesions, either in the nature of a partial or complete vascular obstruction (multiple thrombosis of minute vessels), or of a process allied to multiple embolism, or possibly the presence of some irritant agent in the circulation may stimulate the endothelium cells of the walls of the vessels, and give rise to extravasation of lymph cells, and perivascular changes at certain spots, and as a result patches of degeneration may follow..." [Critical digest, 1896]. This interest in the brain vessels in MS seemed to have evaporated, about the time of Louis Pasteur, with the recognition that neuroparalytic accidents could arise after injection of rabies vaccine prepared from the spinal cord of rabbits. Then Rivers and colleagues induced acute encephalomyelitis in monkeys by nervous tissue immunisation [Rivers T.M. et al, 1935] and soon after, experimental allergic encephalomyelitis (EAE) was generated with the introduction of Freund adjuvant [Freund J. et al, 1947]. Most of the MS researchers focussed their interest on the myelin and on the autoimmune theory of MS; the brain vessels were forgotten, few tried to revive them but were mostly unnoticed [Putman T.J., 1937]. As MS researchers were preoccupied with the viral theory of MS in the sixties and seventies, it is only recently that the brain vessels have regained the place they deserve alongside the myelin-autoimmune and viral theories of MS.
Histopathological considerations of the brain vessels in relation to the MS lesions.

As already discussed, the MS lesions are strikingly perivascular, sometimes extending over a considerable distance along the course of a vein or sometimes as Dawson's fingers. Cellular infiltrates or cuffings don't always arise in proximity to myelin as MS lesions are sometimes present in the grey matter and, as periphlebitis retinai, around the veins of the retina which is devoid of myelin. NMR studies with Gadolinium-DTPA show that in the relapsing-remitting form of the disease, disruption of the BBB is an early event and that the MS lesions develop in a centrifugal fashion. The disruption of the BBB appears to be reversible in serial NMR studies [Kermode A.G. et al, 1990] but is still present in established lesions: T. Broman demonstrated that perfusion of the brain with trypan blue shortly after death caused staining in the MS plaques [Broman T., 1947]. In addition, in some very early lesions the lymphocytic infiltrate is confined to the cerebral vessel wall and in the typical MS lesions the perivenular infiltrate seems to accumulate in the Virchow-Robin space.

What happens to the vessel wall in 'active' MS plaque is less well documented. Signs of vessel wall damage has been observed in some cases. They range from 'recent perivenular haemorrhages', 'venular thrombosis' and 'intramural fibrinoid deposition' in about 2-5 % of cases to 'perivenular iron deposition' - sign of old hemorrhage - in about 30 % of cases [Adams C.W.M., 1989]. In chronic MS lesions, the prolonged effect of inflammatory or traumatic damage to the vessel leads to the collagenisation and marked scarring even to the extent of collagenous degeneration or hyalinisation of the vessel [Adams C.W.M., 1989] [Allen I., 1981].

Electronmicroscopical studies of the vessels in acute MS plaques has shown that the tight junctions appear closed but it is still possible that temporary opening and subsequent closure of the junctions may occur: '...the gate can open and close depending upon who knocks...'[Brown W.J., 1978]. The shape and number of the endothelial cell mitochondria are not modified by the presence of recent MS plaques but the number of pinocytic vesicles is markedly increased in the endothelial cells of the MS plaques [Brown W.J., 1978]. The same findings have been observed in chronic EAE [Hawkins C.P. et al, 1990].
Hypotheses: the involvement of the brain blood vessels in the aetiopathogenesis of MS.

Thrombosis has been linked with the pathogenesis of MS [Putman T.J., 1937] but it is now thought that it is a result rather than the cause of the plaque (for review see [Adams C.W.M., 1989]). In the same context, P. James suggested that the lesions of MS might be due to transient venous occlusion by fat embolism [James P.B., 1982] and this would explain the possible effectiveness of hyperbaric oxygen treatment in MS. However, neither the fat embolism theory nor the effectiveness of hyperbaric oxygen treatment have been confirmed [Oppenheimer D.R., 1982] [Wiles C.M., 1986].

Trauma of the vessels has been put forward by C.M Poser as a possible trigger for MS lesions [Poser C.M., 1896]. He argued that fresh MS lesions have been observed near the needle tract after stereotactic procedures [Gonsette R. et al, 1966][Riechert T. et al, 1968]. Also, the predominance of cervical lesions compared with the rest of the spinal cord may implicate mechanical stresses as a triggering factor for MS lesions: the lateral portion of the spinal cord close to the attachment of the denticulate ligaments were the most common sites of plaques in a study of MS spinal cords [Oppenheimer D., 1978]. However, trauma of the vessels is not sufficient for the genesis of MS: Poser compared MS to the experimental conditions designed by Clark and colleagues when they observed clustering of the EAE lesions (after immunization with spinal cord and Freund's adjuvant) around the previously made electrolytic lesions in the cerebral cortex and white matter of rabbits and guinea pigs [Clark G. et al, 1955] [Bogdanove L. et al, 1957]. However, Poser hypothesised that the cause of alteration of the brain vessels in MS patients may be due to factors other than trauma, for example it may also be generated by viral infection.

Other immunological hypotheses stem from the potential immunological functions of these cells. Post capillary venules are the sites of extravasation of lymphocytes in inflammatory lesions and thus can be compared to some extent with the high endothelial venule of the lymph nodes para-cortex [Kraal G. et al, 1987]. This function seems to arise in part because of the large density of adhesion molecules. In MS these adhesion molecules could be overexpressed due to a number of different mechanisms e.g viral
infection. On the other hand the lymphocytes in MS could be more 'sticky' because of their activation and expression of adhesion molecules [Springer T.A., 1990] and therefore adhere to the endothelial cells. Consequently, the lymphokines generated by lymphocyte activation could induce, in a paracrine fashion, enhanced expression of MHC and adhesion molecules on the surface of the endothelial cells [Pober J.S., 1988]. The same types of mechanisms for the extravasation of lymphocytes can also be put forward using monocytes and macrophages. It has also been proposed that EC may act as antigen-presenting cells for autoimmune antigens (e.g MBP) in MS. It has been shown that peroxidase-labelled MBP injected into the CSF of rabbits was presented at the level of the brain vessels after a period of time [Vass K. et al, 1984]. Cross and colleagues have proposed that a small number of antigen (MBP?) specific T cells could prime central nervous system endothelial cells which could then recruit inflammatory cells in a non specific manner [Cross A.H. et al, 1991]. During the antigen presentation, brain endothelial cells could become target for MBP specific cytotoxic T cells. This has been demonstrated in vitro with rat brain endothelial cells which are able to present MBP to MBP specific T cell lines and be lysed in the process [McCarron R.M., 1991][Risau W. et al, 1990]. The possibility that after destruction of the myelin in optic neuritis some myelin is released into the circulation and then taken up by the EC of the retinal vessels could perhaps explain the occurrence of periphlebitis retinae in some MS patients; this possibility could also explain the lesions in the grey matter. Targeting the early events at the level of the blood-brain barrier could lead to rewarding therapeutic results in patients suffering from MS. Yednock and colleagues have shown that EAE can be prevented by treating the animal with an antibody against α4β1 integrin which is an adhesion molecule expressed on lymphocytes and monocytes [Yednock T.A. et al, 1992].

Reactivation of a viral infection or a low-grade persistent infection of the brain EC in patients suffering from MS is also a very interesting possibility. This could interfere with cell synthesis or induce a local T or B cell response to the virus but also to the EC themselves [Ault K.A. et al, 1979] [Friedman H.M. et al, 1981]. Release of normally hidden antigens, opening up of the BBB as well as the expression of immunological molecules could amplify this immunological response. Moreover it has been shown that EC infection with some viruses increases local procoagulant activity.
[Etingin O.R. et al, 1990]. Very little work has been published on viral infection of brain EC: it has been noted that human brain EC are permissive for infection by human cytomegalovirus which occurs frequently in AIDS patients [Lathey J.L. et al, 1990].

The brain vessels could also be the target for an autoimmune attack without viral infection. Tsakuda and his colleagues have reported the induction of chronic EAE in guinea pigs and in monkeys by immunisation with cerebral endothelial cell membranes [Tsakuda N. et al, 1987 and 1988]. However, this study still needs to be reproduced by other independent research groups. Concerning the humoral arm of the immune response it has been reported that MS patients and patients suffering from HTLV1-associated myelopathy have autoantibodies to endothelial cells [Tanaka N. et al, 1987] [Tsakuda N. et al, 1989, a and b]. Complement induced toxicity of the brain vessels triggered by different factors e.g infection, can be induced with or without autoantibodies to EC. Compston and colleagues have reported the deposition of C9 (part of the membrane attack complex) on the brain vessels of MS plaques [Compston D.A.S. et al, 1989]. Involvement of autoantibodies to EC has been suggested as causal in some 'low grade vasculitis' e.g in connective tissue diseases [Rosenbaum J. et al, 1988] or in systemic lupus erythematosus [Vismara A. et al, 1988].

If injury of EC is causal in MS, demyelination could occur and be perpetuated non specifically by macrophages [Cuzner M.L. et al, 1988], by complement [Scolding N.J. et al, 1989] or even by proteinase from the disrupted myelin sheaths [Chantry A. et al, 1988]. Moreover cytokines secreted by lymphocytes can also mediate demyelination [Owen S.J. et al, 1988].
THE VIRAL THEORY IN MS.

Epidemiology.

Although genetic predisposition has been well established in the cause of MS, many features of the epidemiology of MS implicate one or multiple environmental agents [McDonald W.I., 1986] [Kurtzke J.F., 1980]. Among the possible environmental agents, viruses are on the top of the list; other factors which have been proposed as causal have been extensively reviewed by Acheson [Acheson E.D., 1985] and will not be discussed in the present review.

The increasing prevalence of MS with latitude in both the northern and southern hemispheres has led epidemiologists to believe that environmental factors may play a role in MS [Kurtzke J.F., 1980] and that these environmental factors are influenced by latitude associated factors e.g the degree of sunlight exposure [Acheson E.D., 1985]. This correlation with MS prevalence can be seen in New Zealand [Skegg D.C.G. et al, 1987], Australia [Hammond S.R. et al, 1988] and the United States [Kurtzke J.F. et al, 1979]. Thus, J.F. Kurtzke divided the globe into almost parallel zones of high, medium and low risk following the lines of latitude [Kurtzke J.F., 1980]. There are however many exceptions to this 'latitude rule', for example the unexplained very high MS prevalence in the north of Switzerland (almost as high as in the north east of Scotland) [Compston D.A.S., 1989] and also the relatively high prevalence of MS in Sardinia [Granieri E. et al, 1983].

Epidemiologists have studied migrants from different risk zones and noticed that if one migrated before the age of puberty, one acquired the risk of the country of adoption whereas if one migrated after puberty one retained the risk of the country of origin. This was demonstrated both ways: from low risk to high risk areas [Alter M. et al, 1978] and also from high to low risk countries [Dean G. et al, 1971] [Acheson E.D., 1985]; it was therefore concluded that the environmental agent(s) was likely to have been contracted around the time of puberty. More recently, it has been reported that children of migrants from the West Indies (a low risk country) to the West-Midlands (a high risk area) have the same risk (unlike their parents) of getting MS as the general population of the West-Midlands and thus it was concluded that the role played by environmental agents
may be even more important than the genetic predisposition [Elian M. et al, 1987].

Foci of MS have been reported in Scotland [Sheperd D.I, 1980], in Scandinavia [Kurtzke J.F., 1974] [Kinnunen E., 1984], in the north of Switzerland [Compston D.A.S., 1989] and in Brittany, France [Gallou M. et al, 1983] to cite but a few examples. Small clusters of the disease has also been reported in small villages or areas for example in Mossyrock, Washington [Koch M.J. et al, 1974], in Duxubury [Deacon W.E. et al, 1959], in Mansfield, in Massachusetts [Eastman R. et al, 1973] and in Nova Scotia [Murray T.J., 1976], in an Aberdeenshire valley [Sheperd D.I., 1980] and in Los Alamos, New Mexico, near the plant where the atomic bomb was developed during the second world war [Hoffman R.E. et al, 1981]. An interesting MS cluster was reported by Campbell and his colleagues: 4 of 7 main research workers on swayback, a demyelinating illness of sheep, developed MS like symptoms some months after completing this work [Campbell A.M.G. et al, 1947]. A diagnosis of MS was confirmed in some of the patients when the post mortem brains became available [Dean G. et al, 1985]. It appears also that in familial cases of MS, siblings concordant for the disease often develop the signs of MS during the same year even though they are of different ages [Waksman B., 1986]; these observations point again to common triggering agents.

The most interesting and the most commented report was the 'MS epidemic' of the Faroes islands during and just after the second world war [Kurtzke J.F. et al, 1979]. From the beginning of this century to the second world war, the Faroes, a group of secluded islands, situated between the North of Scotland, Norway and Iceland had only one case of MS recorded. Compared to the neighbouring islands, the Shetlands and the Orkneys, and, also to Norway, the low prevalence of MS in the Faroes never fitted the 'latitude theory of MS'. However, 32 native and resident Faroeses developed MS during and after the war [Kurtzke J.F. et al, 1986]. At the beginning of 1940, British troops occupied the Faroes and stayed defending the islands until the end of the war [Kurtzke J.F. et al, 1986]. It was thus postulated that an environmental agent carried by or accompanying the British soldiers was introduced in the islands and caused this 'MS epidemic' [Cook S.D. et al, 1978] (see the paramyxovirus section). The same phenomenon seemed to have occurred in Iceland after the war [Kurtzke J.F. et al, 1980]. More recently, another 'MS epidemic'
was described in Key-West, a small island south of the Florida coast; this 'epidemic' did not correlate with the risk zones of MS as the Gulf of Mexico is a low risk area [Sheremata W.A. et al, 1985].

Although most of the epidemiological features of MS in favour of an external causal agent are interesting and reasonably convincing, it is fair to acknowledge, first, that the statistical tests used in some studies were stretched to the limit of significance (for example in the migrant studies), and, secondly, that some MS researchers have questioned the validity of some observations: Poser and colleagues had doubts about the 'Faroes epidemic' [Poser C.M et al, 1988, a and b] and others about the 'Key-West epidemic [Román G.C. et al, 1987]. Also, some MS researchers gave a possible genetic explanation to some epidemiological features of MS: the relationship of MS prevalence to latitude could be explained by the distribution of white people of Nordic origin around the globe [Poser C.M., 1987] [Ebers G.C. et al, 1986], and even in New-Zealand [Skegg D.C.G. et al, 1987] or in Great Britain [Swingler R.J. et al, 1986]. Nevertheless, it is paradoxically the genetic twin studies which give the strongest evidence of the involvement of environmental agents in MS. Only 30 % of identical twins are concordant for the disease; in other words, in 70 % of cases, an individual with exactly the same set of genes as a person suffering from MS, will never develop symptoms of MS [Ebers G.C et al, 1986]. However, in a further 10 % - still leaving 60 % - of these monozygotic pairs of twins, the unaffected twin shows signs of MS on NMR [Ebers G.C. et al, 1986]. Interestingly, a concordance study of paralytic polio in twins gave similar results to the MS twins studies (paralytic polio - concordance in monozygotic (MZ) twins: 35.7 %; in dizygotic (DZ) twins: 6 %. MS - concordance in MZ twins: 25.9 %; in DZ twins: 2.3 %) [Herndon C.N. et al, 1951] [Eldridge R., 1987]). This latter study suggests, again, that MS is caused by an environmental agent, probably viral and fairly ubiquitous in the general population, only in individuals with a specific genetic background.
Human and animal demyelinating diseases caused by viral infections: general consideration.

Viral diseases leading to demyelination occur in humans and in animals and can also be induced in laboratory animals. Although some of the mechanisms leading to demyelination are not completely understood in most of these viral diseases, it is nevertheless clear that these mechanisms can be different between diseases and may also be multifactorial within the same disease. Nevertheless, some insight into demyelination caused by viruses has been gained by the study of these viral diseases. Some of the diseases have been modified in laboratory animals to resemble MS more closely and thus alongside the autoimmune experimental allergic encephalomyelitis (EAE) induced by myelin basic protein (MBP), these viral demyelinating illnesses are reasonable models for multiple sclerosis. Many more viruses can cause infection of the nervous system but do not lead to demyelination and thus they will not be discussed in this chapter.

Human demyelinating diseases caused by viral infections.

For a summary of the human demyelinating diseases caused by viral infection and their characteristics see Table 1a.

a) Progressive multifocal leukoencephalopathy (PML).

PML occurs in immunocompromised hosts (e.g. patients with AIDS, leukemia or lymphoma), and is caused by the human papovavirus JC [Richardson E.P., 1988] [Padgett B.L., 1976] and had also been associated with SV 40 [Weiner L.P. et al, 1972]. Multifocal regions of demyelination can be seen in post mortem brains. They become confluent as the disease progresses. The oligodendrocytes are enlarged as well as the astrocytes [Brooks B.R. et al, 1984]. Large number of papovavirus particles can be visualized in the nuclei of oligodendrocytes by electron microscopy [zuRhein G.M., 1967] and virus can be detected occasionally in astrocytes but not in endothelial cells with in situ hybridization techniques [Aksamit A.J. et al, 1985].
<table>
<thead>
<tr>
<th>Disease</th>
<th>Proposed Cause of Demyelination</th>
<th>Lesion Sites</th>
<th>Presence of Virus in Affected Areas</th>
<th>Presence of Affected Disease</th>
<th>Virus</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS DEMYELINATION</td>
<td>MULTIPLE SCLEROSIS SYMPTOMS AND SYMPTOMS OF SPINAL CORD/SPINAL MUSCLE</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
<td></td>
</tr>
<tr>
<td>POST INJECTIONS</td>
<td>ENCEPHALITIS/ENCEPHALOMYELITIS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
<td></td>
</tr>
<tr>
<td>IMMUNE MEDICATIONS</td>
<td>POST INJECTIONS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
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<tr>
<td>IMMUNE MEDICATIONS</td>
<td>IMMUNE MEDICATIONS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
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<tr>
<td>IMMUNE MEDICATIONS</td>
<td>IMMUNE MEDICATIONS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
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<td>IMMUNE MEDICATIONS</td>
<td>IMMUNE MEDICATIONS</td>
<td>SPINAL CORD</td>
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<td>HIV 2 AND HIV 1</td>
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<td>IMMUNE MEDICATIONS</td>
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<td>HIV 2 AND HIV 1</td>
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<tr>
<td>IMMUNE MEDICATIONS</td>
<td>IMMUNE MEDICATIONS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
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<tr>
<td>IMMUNE MEDICATIONS</td>
<td>IMMUNE MEDICATIONS</td>
<td>SPINAL CORD</td>
<td>++</td>
<td>HIV 2 AND HIV 1</td>
<td>HIV 1 AND HIV 2</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1a: Human Demyelinating Diseases**
One possible explanation of the demyelination process in PML comes from the study of transgenic mice. Transgenic mice containing the JC early region, which codes for the T antigen, have been produced [Small J.A. et al, 1986]. These mice develop 'shaking' and the expression of the T antigen correlates with the severity of the 'shaking'. As the T antigen shares some homology with the C-terminal part of MBP, it appears to inhibit competitively the protein kinase phosphorylation of the C-terminal portion of MBP. This blocks the production of myelin and arrests the maturation of oligodendrocytes [Chan K-F.J. et al, 1986]. The T-antigen has been demonstrated in the nuclei of oligodendrocytes of patients with PML [Stoner G.L. et al, 1986] but is not detected in oligodendrocytes of patients with MS [Stoner G.L. et al, 1986].

b) Post infectious encephalomyelitis.

Post infectious encephalomyelitis (PIE) shows an obvious temporal relationship to some acute viral infections particularly measles, chickenpox, as well as smallpox vaccination. It can also be seen following unidentified upper respiratory febrile infections. Children are most commonly affected. PIE usually occurs a few days after the beginning of an acute infectious illness. Fever, headache, vomiting and increasing drowsiness may be accompanied by focal or diffuse signs of brain or spinal cord disease. Death occurs in about 20% of cases and although some patients suffer permanent neurological sequelae recovery can be complete [Matthews W.B., 1985].

The virus is rarely isolated from the brain and the histopathology resembles acute EAE with demyelination and intense perivascular inflammation and thus it is thought that the disease is immune mediated and that the virus does not play a direct role in the pathogenesis. PIE due to measles has been studied extensively. CD4 positive lymphocytes specific for MBP mediate demyelination, at least in part [Lisak R.P., 1977] [Johnson R.T. et al, 1984] [Hafler D. A. et al, 1987]. It is not understood why such pathogenic T cells arise after so many different viral infections. Some have suggested molecular mimicry between a common virus and MBP as possible trigger of autoimmunity [Jahnke U et al, 1985] [Weise M.J. et al, 1988] [Souberbielle B.E. et al, 1991].
c) HTLV-1.

The human T-cell leukemia/lymphoma virus (HTLV-1) is a retrovirus which causes adult T-cell leukemia (ATL) and T cell lymphoma [Yoshida M. et al, 1982] [Poiesz B.J. et al, 1980]. It has also been associated with tropical spastic paraparesis (TSP) [Vemant J.C. et al, 1987] [Cruickshank K. et al, 1989]. In Japan, a similar disease has been described and named HTLV-1 associated myelopathy (HAM) and seems to be the same disease despite some slight differences (see below) [Osame M. et al, 1987].

Clinically, TSP is characterised by a slowly progressive, symmetrical, predominantly upper motor neuron disorder where signs of spastic paraparesis are predominant. Thus TSP lacks remissions typical of MS and also sensory signs are minimal or absent - in HAM the sensory signs have been claimed to be more prominent than in TSP [Osame M. et al, 1987] [Vernant J.C. et al, 1987]. Elevated serum antibodies to HTLV-1 are present in patients suffering from TSP compared to controls [Gessain A. et al, 1985]. High level of antibodies are detected in the patients' CSFs and are produced intrathecally with occasionally oligoclonal banding - the CSF disturbances appear to be more pronounced in HAM than in TSP [Tournier-Lasserve E. et al, 1987] [Osame M. et al, 1986]. Mononuclear cells with large multilobular nuclei in the CSF have also been described in HAM [Osame M. et al, 1986]. Histologically, demyelination and perivascular cuffing by lymphocytes of small vessels of the spinal cord are present. Macrophages as well as plasma cells are also seen in the lesions which are distributed in both white and grey matter. The extent of meningeal inflammation is much more important in TSP/HAM than in MS [Editorial, Lancet, 1988].

As in most (if not all) demyelinating diseases, host factors are very important in the disease (determining occurrence and also progression). The cause of demyelination in TSP/HAM is still unclear: it is not known if the virus has a direct effect on the oligodendrocytes and/or the myelin or if the demyelination is immune-mediated. For example it has been demonstrated in vitro that antibody directed cytotoxicity (against HTLV-1 infected cells) is much higher in symptomatic patients than in asymptomatic individuals [Dalgleish A.G. et al, 1988]. However, the prime suspect for initiating the disease is the HTLV-1 infected lymphocytes and it
is thought that HTLV-1 is not truly neurotropic [Dalgleish A.G., 1991]. In the peripheral blood the main cell infected is the CD4+, CD45 RO+ T lymphocytes [Richardson J.H et al, 1989,b]

d) Acquired immunodeficiency syndrome (AIDS).

HIV produces a variety of neurological conditions but there is still some confusion about the name(s) of the syndromes related to these conditions. Moreover, categorisation may be oversimplistic as seen below. It is generally accepted that the encephalopathy associated with psychiatric symptoms and with a persistent infection of the CNS by the HIV agent is described under the term "AIDS-dementia complex" but has also been named AIDS encephalopathy, AIDS-related-dementia and subacute encephalitis. However it is not yet clear that this clinical syndrome corresponds to a single aetiopathogenetic entity. Pathologically this syndrome is associated with different histopathological features: vacuolar myelopathy, multinucleated cell encephalitis, diffuse pallor of the white matter and aseptic meningitis (although the latter tends to be also associated with an earlier symptomatic infection of the CNS). Central nervous system (CNS) demyelination is a major feature of the first 2 conditions [Shubin R.A. et al, 1989] whereas pallor of the white matter is related to astrocytic hyperactivity. These abnormalities are predominant in the subcortical structures of the brain and of the spinal cord [Price R.W. et al, 1988]. Other neurological syndromes seen in HIV patients are sensory polyneuropathy and dysimmune motor polyneuropathy as well as the infectious neurological complications associated with AIDS e.g PML or cytomegalovirus and cryptococcus infection of the brain.

Some authors have differentiated the 2 pathological conditions, vacuolar myelopathy and multinucleated cells subacute encephalitis on a clinical basis, although these conditions often coexist in the same patient. Vacuolar myelopathy is characterised by paraparesis, ataxia and incontinence [Petito C.K. et al, 1985] [Levy R.M. et al, 1985]. Pathological examination reveals demyelination predominantly in the lateral and posterior columns of the thoracic spinal cord and demyelination appears to result either from swelling within the layers of the myelin sheaths [Price R.W et al, 1988] or from intramyelin vacuoles resembling lipid-laden macrophages [Petito C.K. et al, 1985] [Asher D.M. et al, 1987]. It also appears that HIV is
not directly associated with the vacuolar myelopathy [Price R.W. et al, 1988]. Some have suggested that this condition resembles subacute combined degeneration of the spinal cord resulting from vitamin B12 deficiency but serum B12 levels are normal.

Multinucleated cells subacute encephalitis is characterised clinically by impaired memory and concentration with psychomotor slowing [Navia B.A. et al, 1986, a] and pathologically by abnormalities in the CNS white matter and subcortical structures; the spinal cord is generally less often affected. These abnormalities consist of white matter pallor, microglial nodules and perivascular and parenchymal macrophages, multinucleated giant cells, microglia and lymphocytes [Navia B.A. et al, 1986, b]. These infiltrates are often surrounded by focal rarefaction of the myelin (demyelination) but more commonly by oedema; loss of oligodendrocytes is not characteristic. HIV has been demonstrated in monocytes and multinucleated cells in the regions of demyelination but not in oligodendrocytes [Navia B.A et al, 1986, b].

Oligoclonal immunoglobulins are produced intrathecally in some patients suffering from AIDS-dementia complex but the specificity of these oligoclonal immunoglobulins is not fully known although some have related these oligoclonal bands to HIV proteins using the immunoblotting method but this result has to be confirmed by other CSF characterisation methods [Goswami K. et al, 1991]. There is also an intrathecal synthesis of antibodies to HIV which has not been correlated to these oligoclonal bands. The virus can be isolated in the CSF. The cell types infected in the brain (in vivo) by HIV are still a matter of debate but it is generally accepted that the main cell involved in productive infection is the macrophage and the cells derived from the macrophage i.e microglial cells [Price R.W. et al, 1988] [Kennedy P.G.E. et al, 1989]. Multinucleated cells appear to be induced by cell fusion similar to the syncytial pathology described for HIV-1 infected CD4 positive cells in culture [Price R.W. et al, 1988]. It is not clear whether or not other cell types are infected in vivo i.e endothelial cells [Willey C.A. et al, 1986], astrocytes, oligodendrocytes and neurons [Gyorkey F. et al, 1987].

The occurrence and the progression of the AIDS- dementia syndrome appear to be influenced by host/genetic factors [Price R.W. et al, 1988] and immunosuppression overall appears to be associated with increasing neurological signs. The CNS signs may
relate as much to the inflammatory changes and 'bystander effects' as to the cytopathic effect of the virus itself. It is not known if the CNS disease is due to specific neurotropic HIV strains in a patient as it is well known that several isolates from a single patient may differ from each other [Saag M.S. et al, 1988] [Fisher A.G. et al, 1988]. The cause of the demyelination (as well as the other pathological signs) is not understood and as already described above the demyelination associated with vacuolar myelopathy is likely to be of different aetiopathogenesis to the demyelination occurring with multinucleated cells subacute encephalitis. However it is likely that in both cases soluble factors are responsible, at least in part, for the myelin attack. These (e.g cytokines or enzymes) could be secreted by infected cells (e.g macrophages), by the inflammatory cells (e.g T cells) or by activated cells in the vicinity of the lesions (e.g endothelial cells).

**Viral demyelinating diseases in animals.**

Most of the following diseases share interesting characteristics: they are biphasic with a stage of acute encephalitis followed by a stage of chronic demyelination. The virus is present in the brain of the animals, particularly in the white matter and the lesions are multifocal (for a summary of the different characteristics of the animal demyelinating diseases mentioned in the text, see Tables 1b)
<table>
<thead>
<tr>
<th>Proposed Cause of Demyelination</th>
<th>Lesions in Affected Areas of the Nervous System</th>
<th>Presence of the Virus in the Afferent Region</th>
<th>Affected in</th>
<th>Virus</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T cells and macrophages</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>CD8+ T cells and macrophages</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>DTH to neural myelin</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>IFN-gamma +</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>IFN-gamma -</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
<tr>
<td>IFN-gamma +</td>
<td>Demyelination</td>
<td>Present</td>
<td>C-pol</td>
<td>(CJD)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1b: Animal demyelinating diseases**
a) Theiler's murine encephalomyelitis virus.

Wild type Theiler's murine encephalomyelitis virus (TMEV), a picornavirus, usually produces an asymptomatic enteric infection in mice, and only rarely encephalomyelitis. One strain of TMEV, Daniel's (DA), when injected intracerebrally, produces a biphasic neurologic disease in Swiss mice without significant neuronal necrosis [Lipton H.L., 1975]. Nine to twenty days after inoculation the mice develop encephalomyelitis and the survivors, one to five months later, develop a mild gait disturbance in conjunction with the occurrence of demyelination in areas of intense mononuclear inflammation. Mice strains vary in their degree of susceptibility to TMEV [Lipton H.L. et al, 1984] [Yamada M. et al, 1991] and it is the D region (MHC class I) which determines this [Rodriguez M. et al, 1986].

During the acute phase of the disease, TMEV can be found in neurons and oligodendrocytes but it is only present in oligodendrocytes cells during the late phase [Brahic M. et al, 1981] [Rodriguez M. et al, 1983] and virus can also be detected in macrophages [Yamada M. et al, 1991]. In nude mice, viral mRNA has been detected in brain endothelial cells [Zurbriggen A. et al, 1988]. It appears that the demyelination is due to a delayed-type hypersensitivity (DTH) T cell response against persistently infected oligodendrocytes [Clatch R.J. et al, 1985], and attempts to demonstrate autoimmune demyelination by adoptive transfer of lymphocytes from diseased demyelination by adoptive transfer of lymphocytes from diseased mice into genetically compatible mice that have not been infected with TMEV have failed [Barnabo R.L. et al, 1984]. However, as athymic nude mice infected with TMEV can develop demyelination, albeit to a lesser extend than the other mice, TMEV could in part cause demyelination by direct cytopathic effect on the oligodendrocytes or through humoral immune response. Molecular mimicry between components of oligodendrocytes and the Theiler's virus has been implicated as a possible cause of myelin damage. Shared molecular structure has been demonstrated between the VP-1 glycoprotein of the virus and galactocerebrosides of the myelin [Yamada M. et al, 1990].
b) JHM strain of mouse hepatitis virus.

JHM virus (JHMV), the neurotropic strain of mouse hepatitis virus, is a coronavirus which produces an acute, diffuse encephalomyelitis with patchy demyelination in mice and rat [Weiner L.P., 1973]. Lesions develop 5 to 7 days after intracerebral inoculation of the virus and inflammation and necrotic lesions are found in the white and grey matter. The degree of demyelination depends on the age and the strain of the animal [Stohlman S.A. et al, 1978]. JHMV is present in the oligodendrocytes but there is apparently no anatomical relation between the inflammation and the demyelination, thus a direct effect of the virus seems to be responsible for the demyelination [Shubin R.A. et al, 1989].

After this acute episode, the animal can recover and go on to develop chronic recurrent demyelination [Stohlman S.A. et al, 1981]. Temperature sensitive mutants of JHMV cause chronic demyelination with minimal encephalitis [Knobler R.L. et al, 1982] and a clinical relapsing form of the disease [Wege H. et al, 1984]. In this situation, the immune system is likely to play a role in the demyelination as demyelination can be transferred from infected to naive rats by adoptive transfer of the lymphocytes, following *in vitro* stimulation with MBP [Wanatabe R. et al, 1983] [ter Meulen V.T., 1988]. The chronic demyelination may also be due to a persistent attenuated infection of oligodendrocytes [Erlich S.S. et al, 1987].

c) Canine distemper.

Canine distemper virus (CDV) is a paramyxovirus related to measles which produces acute or chronic demyelinating disease in dogs (as well as an associated respiratory infection) depending on the strain of the virus and on the age of the dog [Raine C.S., 1974] [Russell W.C., 1983]. Demyelination can apparently occur in the absence of inflammatory cells: perivascular inflammation can be seen in infected brains in association with demyelination but more often in association with areas in which clearance of the virus has taken place. Thus, this inflammatory reaction may represent a secondary response to demyelination [Bollo E. et al, 1986].

Oligodendrocytes appear not to be infected by the virus [Zurbrigger A. et al, 1987] but it has been shown that other cells of the CNS are infected e.g astrocytes [Mutinelli F. et al, 1989] and it appears that
infection of the endothelial cells of the blood-brain barrier is an early event in the disease process [Axthelm M.K. et al, 1987]. However, the role of the humoral response is not clear in the demyelination process as antibodies could act upon CDV-infected oligodendrocytes and/or myelin, alone or in association with complement.

In mice a chronic neurological disease has also been established with a mouse-adapted strain of CDV. Lethality of the infection was found to be mouse-strain-dependent. However mice of resistant strains, notably the SJL strain, were able to survive the acute infection but after a period of clinical normality showed signs of subacute CNS disease with gait disturbances. Histopathological analysis of the brain and spinal cord revealed significant perivascular mononuclear infiltrate and meningitis. However, unlike CDV infection in the dog, demyelination was not seen [Lyons M.J. et al, 1980]. Moreover, in the chronic phase of the mice infection, CDV was not detected in the brain unlike in the dog where CDV persists and retains the capacity to undergo complete replication [Summers B.A. et al, 1983]. CDV has also been studied in an hamster model and it is clear that different strains of the virus can produce very different disease syndromes [Cosby S.L. et al, 1981]

d) Measles virus (MV).

MV can produce a chronic relapsing myelitis in hamsters when injected intracerebrally in very young animals. The difference between this disease and the other animal viral demyelinating diseases is that there is no acute episode. The onset of the myelitis occurs 5 to 50 weeks after the inoculation. In most animals, pathological changes are confined to the spinal cord and are characterised by mononuclear infiltration with gliosis and widespread demyelination and necrosis. The virus cannot be detected by immunofluorescence in the brain of the hamster [Carrigan D.R., 1980]. A chronic relapsing encephalomyelitis in hamsters which bears some similarity to MS has been described [Carrigan D.R., 1987].

Liebert and ter Meulen have also developed a rat model system where the strain of the animal influences the course of the neurological disease [Liebert U.G. et al, 1987]. The same research group showed that in one strain of rat the persistent measles virus
infection resulted in the induction of a cell-mediated autoimmune response against myelin basic protein [Liebert U.G. et al, 1987, b]. ter Meulen and colleagues have used the neurotropic strain of measles CAM/RBH to induce a subacute encephalomyelitis with a 20% success rate. In Lewis rat, the virus cannot be isolated although the antigens of the virus can be detected suggesting that the virus cannot be fully assembled. Cell mediated immunity against MBP is detectable and the encephalomyelitis is transferred to naive animals by lymphocytes from diseased animals. In the CSF, intrathecal production of anti-measles antibodies can be detected but there is no oligoclonal IgG [ter Meulen V.T., 1988]. Rats infected with this virus are prone to develop EAE when immunised with a non-encephalitogenic peptide of MBP suggesting again that regulation of the immune system has a important role to play in the demyelination in these measles-infected rats and the measles virus appears to act merely as a trigger for this autoimmune reaction [Liebert U.G. et al, 1989].

e) Semliki Forest virus (SFV).

SFV is a non-human pathogenic alphavirus and produces demyelination in mice when inoculated intracerebrally [Chew-Lim M. et al, 1977]. Demyelination occurs in conjunction with an inflammatory infiltrate and is immune-mediated [Fazakerley J.K. et al, 1983]. It does not occur in immuno-incompetent mice or immunosuppressed mice despite the presence of the virus in the brains of these animals. Moreover, reconstitution of SFV-infected immuno-incompetent mice with normal spleens leads to demyelination [Fazakerley J.K. et al, 1987, a and b].

In the rat, it has been demonstrated that viral strains are critical: the neurovirulent strain L10 causes extensive neuronal damage whereas the avirulent strain A7/M9 induces infection of oligodendrocytes and demyelination which is likely to also be immune-mediated [Atkins G.J. et al, 1990].

f) Herpes simplex virus type 1 (HSV-1).

HSV-1 can produce meningitis and encephalitis in man. After oral-facial inoculation in mice, lesions of demyelination in association with an inflammatory mononuclear infiltrate in the
brainstem adjacent to the trigeminal root entry zone (TREZ) can be induced [Kastrukoff L. et al, 1989]. Multifocal demyelination of the CNS has been induced in mice with HSV-1 [Kastrukoff L. et al, 1989]. This condition depends on the murine strain used e.g PL/J is the most susceptible strain. After lip inoculation with HSV-1, PL/J mice develop demyelination at the TREZ as in other susceptible strains of mice. However, in this strain of mice, lesions appear in other parts of the brain later. The demyelination is associated with mononuclear inflammation where macrophages are predominant. The virus is not detected in the multifocal lesions unlike the unifocal lesions of the TREZ where HSV-1 is detected in neurons, astrocytes and oligodendrocytes [Kastrukoff L. et al, 1989] [Townsend J.J., 1983].

The mechanisms mediating demyelination are still unclear but probably involve both the immune system and a direct cytotoxic effect of the virus. [Townsend J.J. et al, 1979] [Kristensson K. et al, 1982]. These mechanisms appear to be different between the unifocal and the multifocal disease.

g) Visna.

Visna is a lentivirus of the retrovirus family which produces pneumonia (the disease is called 'maedi' in Iceland) and/or a chronic progressive, although occasionally relapsing-remitting, myelopathy in sheep (the disease is then called visna) [Weinshenker B. et al, 1989]. Visna has some clinical and biological parallels to MS e.g. age dependence, genetic determined resistance, and increased intrathecal IgG with oligoclonal bands in the CSF [Weinshenker B. et al, 1989]. Pathogenically, demyelination occurs in 2 phases in both the spinal cord and the brain: during the initial phase, demyelination is located in regions of inflammatory infiltrates with relatively little tissue necrosis, and during the later phase, demyelination occurs in conjunction with necrosis of both grey and white matter [Pertusson G., 1976].

The visna virus undergoes antigenic drift during the persistent type of infection in sheep [Narayan O., 1977] [Haase A.T., 1986] [Clements J.E. et al, 1988]. It can be isolated from the CNS and the blood of affected animals. Then as the disease progresses, the virus appears to be confined to mononuclear cells (primarily to
monocytes and macrophages and can be detected in the blood, CNS and CSF in these cells. This correlates with the period of intense humoral immune response [Cruickshank K. et al, 1989]. It appears that immunopathological mechanisms, especially soluble factors released by infected macrophages play a role in the genesis of demyelination [Shubin R.A., 1989] [Cruickshank K. et al, 1989] but perturbation of the function of oligodendrocytes mediated by a non productive infection of these cells by the visna virus has not been excluded as a possible cause of myelin damage.

h) Caprine arthritis encephalitis

Caprine arthritis encephalitis virus is also a retrovirus from the lentiviridae family. In young goat a progressive paraplegia occurs and pathologically the lesions are similar to those of visna. However the antibody response to this virus is absent in the affected animal unlike in cases of visna [Narayan O. et al, 1985] [Cruikshank K. et al, 1989].

Immune response against viruses in MS.

a) Humoral response.

Numerous surveys of antibody titers specific to viruses have been carried out in MS patients compared to controls [Cook S.D. et al, 1980]. Measles has been studied extensively after Adams and Imagawa first demonstrated that patients with MS when compared to controls had higher titers of serum antibodies specific to the measles virus [Adams J.M. et al, 1962]. This observation has been repeated many times in the serum and in the CSF, with different methods. Moreover the humoral immune response is directed against more than one measles protein [Norrby E., 1978]. The measles antibody titer elevation is modest - about 2 fold higher than in the controls. It appears also that in most cases measles virus antibodies are being produced intrathecally as alterations in serum/CSF measles antibody ratios have been found in a number of studies [Haire M., 1977] [Norrby E. et al, 1974] [Vandvik B. et al, 1975] [Salmi A.A., 1972]. However, no relationship has been found between measles titer and disease activity [Norrby E., 1978] [Haire M., 1977]. Interestingly,
the measles serum antibody titer of MS patients' siblings without
evidence of MS also tends to be higher than that of controls [Henson
1973].

Other viral antibodies serum titers have been found elevated in
MS populations compared to controls (although this is less consistent
than for measles) for example with vaccinia, rubella, herpes simplex,
mumps, canine distemper, Epstein-Barr virus, adenovirus [Cook
S.D. et al, 1980] or HTLV-1 (see below). Thus, it is thought that
higher titers of serum antibodies to measles and to other viruses may
reflect a non specific dysregulation of the immune system (either
secondary or primary to the disease process).

In the CSF of MS patients intrathecal production of antibodies
to viruses other than measles have been detected for example with
vaccinia, herpes simplex, rubella, mumps and other viruses (for
reviews see [Cook S.D., 1980] and [Walsh M.J. et al, 1983]).
Furthermore, multiple viral antibodies may be detected in the CSF
from the same patient and fluctuations in titers may occur [Salmi A.
et al, 1983] [Arnadottir T. et al, 1979]. Salmi and colleagues
measured the intrathecal antibody synthesis to 16 different viruses in
the CSF of 30 patients with MS and 29 neurological controls without
demyelination. They found that, apart from 2 MS patients, all
showed intrathecal synthesis against more than one virus (usually
about 10 different viruses), and, only 3 controls had antiviral
intrathecal synthesis but against only one virus. In the MS group the
viruses that were over-represented were measles, rubella,
parainfluenza 1 and 3, herpes zoster and mumps [Salmi A. et al,
1983]. This multiple intrathecal anti viral response is interpreted as
being the sign of a non specific stimulation of immunocompetent
cells (B cells and plasma cells) trapped beyond the BBB in MS
brains. In the same way, local synthesis of anti-measles and anti-
rubella antibodies has also been demonstrated in the synovium of a
rheumatoid arthritis patient [Vandvik B. et al, 1977]

The antiviral response has never been convincingly related to
the CSF oligoclonal IgGs which can be detected in over 90% of
definite MS cases. For example a small proportion of the measles
antibodies can be associated with oligoclonal banding but most of the
anti-viral response is of polyclonal nature. Moreover measles
antibodies account for a minor proportion of the total CSF IgGs
[Vartdal F. et al, 1982]. Investigation of the CSF antibodies specific
to the paramyxovirus SV5 will be presented as part of the work in this thesis and background to this work is discussed subsequently in this chapter.

An IgM response (indicative of a primary response or of persistent infection) to measles as well as anti-mumps and anti-varicella IgM can be detected in the CSF of patients suffering from MS [Chodi F. et al, 1987]. This was again interpreted as a sign of non specific intrathecal stimulation in MS brains.

In conclusion, the antibody specificity of the majority of the CSF IgGs produced intrathecally in patients suffering from MS has not been defined; a variable small fraction of these IgGs are found to be virus - specific. It is however possible that some CSF IgGs are directed against an unknown antigen involved in the cause of MS (viral or non-viral) even though most of the CSF IgGs are probably due to non specific intrathecal stimulation. On the other hand, it is possible that the cause of MS is different between patients and that one virus may induce MS in one individual, depending, for example, on the HLA status of this individual, whereas another virus may be the causative agent of MS in another person.

b) Cellular immune response.

The cellular immune response to viruses has been much more difficult to evaluate in MS patients and their controls than the humoral immune response. This may be due to the difficulty of purification of viruses and viral proteins for T cell assays but also due to the incomparability of the techniques used to measure such a response. The antiviral T cell response has been measured by multiple methods especially T cell proliferation assays (an indication of the T helper response), migration inhibition (more difficult to interpret) and cytotoxicity assays (an indication of the T cell cytotoxic response). Even skin tests (an indication of the delayed type hypersensitivity response) have been used. Numerous viruses have been tested - for example measles, mumps, parainfluenza virus, vaccinia, rubella, herpes simplex and cytomegalovirus (CMV) [Cook S.D. et al, 1980]. Overall, the results of the past decades are inconclusive as the reported T cell responses of MS patients when compared to controls have been greater, less or the same with most of the viruses tested.
The techniques of cytotoxic lymphocyte (CTL) assays have recently improved with the recognition of HLA restriction. Target cells used in CTL assays are autologous (of the same HLA as the patient's CTL) whereas in the early days some of the cytotoxicity assays were done with heterologous target cells e.g HELA cells infected with measles. Jacobson and his colleagues have studied the CTL response to measles in patients with MS. First, they demonstrated that in the measles system a high proportion of CTL were HLA Class II restricted and were CD 4 + [Jacobson S. et al, 1984 and 1989]. This was at odds with the concept that CTL must be Class I restricted. Other viral systems however also showed this predominance of class II restricted CTL response e.g herpex simplex virus [Schmid D.S., 1988]. Interestingly, it has also been demonstrated that some CTL clones specific for measles viral proteins were HLA DR 2 restricted in some MS patients [Richert J.R. et al, 1986] (HLA DR 2 allele being associated with multiple sclerosis predisposition [Compston D.A.S., 1986] and thus it was suggested that this anti-measles response may be relevant in the MS process). In addition, the generation of specific CTLs against multiple measles proteins was impaired in a number of selected MS patients [Dhib-Jalbut S., et al, 1989] and the authors suggested that this was due to a persistent infection of measles in MS patients. However, these observations were done on selected patients and thus need to be confirmed to be considered relevant to MS in general.

Natural killer (NK) cell activity in multiple sclerosis patients has been found to be decreased by a number of research groups [Benczur M. et al, 1980]. The explanation for this phenomenon is unknown. Some have suggested that it is a sign of a persistent viral infection.

**Virus isolates in MS and transmissibility of the disease.**

During the last 50 years, it has been claimed that a number of viruses have been isolated from multiple sclerosis patients or have been visualised by different techniques (by electron-microscopy (the virus itself), by 7 (the viral proteins) or by in situ hybridization (the viral genome)). In his review of viruses and multiple sclerosis T.J. Johnson listed the viruses which had been

Retroviruses have now been added to this list (see below). No one has claimed that these viruses are causative but only that they are associated with MS. However, none of these observations has been reproduced by other independent research groups.

Using in situ hybridization techniques, Haase and his colleagues demonstrated that viruses (or at least part of their viral genomes) can reside in the CNS even in normal people and these viruses were also seen in MS brains e.g. measles [Haase A.T. et al., 1981 and 1984] or herpes simplex type I [Fraser N.W. et al., 1981]. Thus, description of viral genomic material in MS tissue has to be taken cautiously.

No one has been able to transmit a MS-like disease from MS tissue to an animal. However this does not mean that a virus is not involved in MS as it has already been seen above that strains, age and even routes of inoculation of viruses are critical factors in the induction of viral demyelinating diseases. Moreover, HIV for example does not induce AIDS in animals other than man, not even in monkeys which apparently have identical CD4 molecules (receptor of the HIV virus) to man [Wanatabe M. et al., 1991]. The reason for the lack of disease in the animal is a matter of debate [Dalgleish A.G., 1992]. In the same way, HIV does not induce a neurological disease in monkeys despite a persistent infection with prolonged viraemia [Fultz P.N. et al., 1986]. Another point to consider is that the virus may have disappeared from the patients suffering from MS at the time of disease. Epidemiology of MS suggests that the environmental agent is contracted 10 to 20 years before the clinical onset of the disease [Rudge P. et al., 1991][Rudge P., 1991].

The question remains whether or not the agent(s) of multiple sclerosis is transmissible between humans. This question is extremely difficult to answer. For example, familial cases are well described.
but it is difficult to dissociate the genetic factors from the environmental factors which are both likely to be shared or be transmitted between members of a same family. It appears that MS is not 'passed' between husband and wife [Acheson E.D., 1985] but it is thought that the environmental agent(s) is likely to act around puberty before marital age. Kurtzke and Hylldested have suggested that in the Faroes epidemic, there were in fact three waves of MS and that the second and third waves were due to the transmission of the external agents from affected but asymptomatic Faroes inhabitants to other unaffected islanders [Kurtzke J.F. et al, 1986]. In the Key-west epidemic, an usually high number of nurses has been found in the group of patients suffering from MS and was interpreted as a consequence of a possible transmission of the 'MS factor' from patients to nurses [Sheremata W.A. et al, 1985].

Apart from the agents isolated from tissue of patients with MS, numerous viruses other than members of the paramyxoviridae family have been postulated as being the cause of multiple sclerosis for example herpes virus [Koprowski H. et al, 1979] [Martin J.R. et al, 1988] [Bergstrom T et al, 1989], Epstein-Barr virus [Operskalski E.A. et al, 1989] or poliovirus [Poskanzer D.C. et al, 1963]. Some have also postulated that common viruses like measles and rubella may induce MS if they are contracted at a specific period in life for example around puberty [Sullivan C.B. et al, 1984] [Compston D.A.S. et al, 1986].

Before reviewing the relationship between paramyxoviruses (the subject of some of the work presented in this thesis), one family of viruses namely the retroviruses will be discussed in more detail as there has been a sustained interest in retroviruses in the context of MS during the past 10 years. Two human retroviral diseases which induce demyelination have been described - AIDS and TSP. Also, a number of animal retroviral diseases are associated with demyelination: visna and caprine arthritis encephalitis.

**Retroviruses and MS.**

In 1985 Koprowski and his colleagues implicated HTLV-1-like virus as an aetiologic agent in MS after they identified antibodies to the p24 protein of HTLV-1 in the CSF of patients from Key West and Sweden. They also found HTLV-1 nucleotide sequences in the leukocytes derived from interleukin 2 expanded
lymphocytes from 4 out of 8 MS CSF samples using the polymerase chain reaction (PCR) technique. The hybridization stringency conditions were, however, low [Koprowski H. et al, 1985]. In addition one group in Japan reported the detection of antibodies to HTLV-1 in a quarter of patients with MS [Ohta M. et al, 1986]. However, subsequent studies looking at the prevalence of anti-HTLV-1 antibodies or at the presence of genomic material from HTLV-1 in the lymphocytes, monocytes or brains of MS patients were unsuccessful [Hauser S.L. et al, 1986] [Karpas A. et al, 1986] [DeRossi A. et al, 1986] [Gessain A. et al, 1986] [Rice G.P.A. et al, 1986] [Kuroda Y. et al, 1987] [Swingler R.J. et al, 1987] [Birnbaum G. et al, 1988] [Bangham C. et al, 1989] [Richardson J.H. et al, 1989] suggesting that the original positive result may have been related to contamination during PCR processing.

Nevertheless, retroviruses as well as paramyxoviruses (see below), are still interesting possible aetiological candidates in MS. They are characterised by a long period of incubation or latency as epidemiological studies of MS suggest for the causal agent. Moreover, latency can be broken by immunological stimuli which are known to be present in MS patients. It has been hypothesised that a still unknown retrovirus could be the cause of MS, and, that this agent will be detected when the right conditions of culture or for example in situ hybridisation can be found [Weinshenker B. et al, 1989] [Perron H. et al, 1989 and 1991]. It is worth remembering that a retrovirus was first incriminated as the cause of AIDS after the demonstration that 30% of patients with AIDS had antibody reactive to cells infected with HTLV I in immunofluorescence assays [Weinshenker B et al, 1989] [Gallo R.C. et al, 1985].

Since Koprowski and colleagues’ report, there have been a number of scientific communications that indicate that retroviruses may be present in MS tissue. For example, Kam-Hansen and colleagues have described a cytopathic effect and the detection of reverse transcriptase activity in the culture of blood mononuclear cells of some patients with MS [Kam-Hansen S. et al, 1989]. Others have detected retroviral genomic material in post-mortem MS brains [Reddy E.P et al, 1989] or in mononuclear cells of some patients with MS e.g sequences homologous to the p19 gag protein [Sandberg-Wolhein M. et al, 1988] or sequences related to the pol proteins [Greenberg S.J. et al, 1989]. The conclusions of all these observations was that a retrovirus different but related to HTLV I
may be associated with some MS cases. More recently, Perron and his colleagues claim that they have isolated a retrovirus from the CSF of one MS patient [Perron H. et al, 1989]. This retrovirus is not a known retrovirus. The CSFs of 2 other MS patients had also antibodies against this virus whereas 2 normal controls did not show any reactivity [Perron H. et al, 1991]. The same group has also reported the presence of antibodies to fragments of retroviral reverse transcriptase in a higher proportion in the sera of patients with multiple sclerosis compared to neurological controls. Comparing the reactions of the positive sera against other proteins of known human retroviruses, they concluded that the reaction observed may have been due to cross reaction against an unknown retrovirus which is likely to be related to the lentivirus family [Perron H. et al, 1991,b]. Other groups have also recently visualised retrovirus-like particles in MS lymphocyte cultures [Haar S. et al, 1991]. However, it is not clear if these retroviruses are endogenous and merely innocent bystanders. Endogenous retroviral DNA is widespread in humans and animals [Larsson E. et al, 1989]. For example, retrovirus-like particles have been observed budding from normal placental trophoblasts [Johnson P.M et al, 1990].

**Paramyxoviridae and MS.**

The viruses of the Paramyxoviridae family have a special place in the long history of viruses implicated in MS. Some of these viruses (MV, CDV and SV5) have been implicated in neurological diseases in both man and animals. As SV5 is particularly relevant to this thesis, a special section of this chapter will be devoted to it.

The paramyxoviridae family is subdivided into 3 genera: 1. the morbilliviruses e.g measles virus (MV), canine distemper virus (CDV), 2. the pneumoviruses e.g respiratory syncitial virus (RSV)) and 3. the paramyxoviruses proper e.g mumps, parainfluenza viruses (PFV) and simian virus 5 (SV5). The viruses of this family have a single strand of negative sense RNA (the genomic viral RNA has to be transcribed in order to act as messenger RNA) and at least 6 virus-coded polypeptides [Russell W.C., 1983]. Some of these viruses can establish persistent infections *in vitro* and *in vivo* [Randall R.E.R. et al, 1990]. One other important property of these viruses is that they are enveloped viruses and are released from the infected cells by 'budding'. Before viral 'budding', viral membrane proteins
are inserted in the plasma membrane of the host cell. It appears that host membrane proteins are displaced and viral proteins agglomerate into patches. The nucleocapsid binds to specific viral protein signals on the membrane viral proteins and the virus extrudes from the cell. In the process host lipids and glycolipids are incorporated into the viral membrane. However, host proteins are not included in the viral membrane [Roizman B., 1990] [Kingsbury D.W., 1990].

a) Measles.

MV has already been covered in the preceding sections of this chapter. Briefly, animal models of demyelinating diseases caused by measles have been described. Patients suffering from MS have higher titers of antibodies to MV in their serum and CSF than normal controls. The cytotoxic T cell response to MV appears to be defective in some MS patients and thus it was proposed some MS patients may harbour a persistent MV infection.

MV or MV-like particles have been detected in the jejunum of some MS patients and not in their controls [Pertschuk L.P. et al, 1976] [Prasad I. et al, 1977] [Ebina T et al, 1979]. These observations were however not confirmed [Woyciechowska J.L. et al, 1977] [Kingston D. et al, 1977] [Fraser K.B. et al, 1977] but no recent work has been done in this domain with molecular biological techniques. MV genomic material has been detected in some post-mortem brains of MS patients (but also in normal controls) by the in situ hybridization technique [Haase A.T. et al, 1984]. However, Cosby and colleagues claim that they were able to detect MV genomic material only in MS brains and not in the control brains [Cosby S.L. et al, 1989].

In more general terms, Alford and colleagues also hypothesised that measles and other common viruses may share some amino acid sequence in common with the myelin proteins (MBP [Jahnke U. et al, 1985] and proteolipid protein (PLP) [Shaw S.Y. et al, 1986]) and this molecular "mimicry" may induce an autoimmune response to myelin in some individuals. They also hypothesised that measles vaccination should in this case lead to a decrease in prevalence of MS in the near future [Alvord E.C et al, 1987]. In the same way, Randall and Russell commenting on the T cell crossreactivity among the paramyxoviridae, for example between 2 unrelated members of the family such as mumps and RSV [Ziola B. et al, 1988], suggested that infections with different paramyxoviruses
may induced cross reactive T lymphocytes which may also cross react with components of the brain [Randall R.E.R. et al, 1990]. A yet unknown virus of the paramyxoviridae responsible for MS could in the same way cross react with other members of the family and induce some of the changes seen in MS patients with MV and with the other related viruses.

b) Canine distemper virus (CDV).

CDV is a virus closely related antigenically to MV [Imagawa D.T. et al, 1960]. CDV is a canine virus which can induce demyelination, and, in laboratory animals, it can lead to a chronic relapsing demyelinating illness (see the section on viral demyelinating diseases in animal in this chapter). CDV was first implicated in MS after Cook and his colleagues observed the occurrence of multiple sclerosis in 3 members of a family 2 or 3 years after the family pet dog suffered from an acute encephalopathy which was thought to have been caused by CDV. They then found in a case control epidemiological study that individuals suffering from MS had more indoor exposure contact with dogs or cats than the control population [Cook S.D. et al, 1977]. During the same period, W. Chan, analysing the geographical distribution of MS and the increase incidence of the disease in families, postulated that MS may be caused by a canine vector, perhaps transmitted by an agent in urine [Chan W.W.C., 1977].

The Faroe epidemic gave more impetus to this possible association of CDV with MS when Cook and his colleagues discovered that a large proportion of dogs in the islands succumbed to a CDV-like disease and particularly to the more virulent, neurologically associated form known as "hard pad". They correlated this CDV-like epidemic to the introduction into the islands of guard dogs by the British troops [Cook S.D. et al, 1978] [Russell W.C., 1983]. The same relationship with CDV and MS was postulated by Cook et al in other 'MS epidemics' as in Iceland [Cook S.D. et al, 1980, b] or in Key-West [Cook S.D. et al, 1987].

The relationship between CDV and MS is however far from demonstrated. For example, many epidemiological studies have not found the association between pet contact and multiple sclerosis sufferers [Read D. et al, 1980] [Cook S.D et al, 1985]. However, it is important to stress that biases (positive or negative) may be introduced in these retrospective studies and a significant difference

In conclusion, despite some interesting epidemiological data between CDV and MS, there is no biological demonstration that CDV is a causative agent of MS. However, it is still possible that most of epidemiological features of MS related to CDV are in fact due to a yet unknown canine virus. In this connection SV5 is an interesting candidate.

c) Simian virus 5 (SV5).

SV5 is a paramyxovirus closely related to but different from parainfluenza 2 (PF-2) and to a lesser extent to mumps [Randall R.E.R. et al, 1988, a]. SV5 is capable of producing persistent infections in vitro and in vivo [Randall R.E.R et al, 1990]. It was first isolated in monkey but seems to be able to infect across species as it has been isolated in dogs and cats and can be used experimentally to infect mice and hamsters [Randall et all, 1988, b and 1990]. It appears that SV5 only causes mild upper respiratory symptoms in animals. It has also been suggested that SV5 may cause widespread inapparent infections in humans [Hsiung G.D., 1972] [Randall R.E.R, et al, 1987]. Nevertheless and despite its name, epidemiologically SV5 is considered to be a canine virus.

In 1978, Mitchell and colleagues reported the isolation of SV5 from the bone marrow aspirates of multiple sclerosis patients [Mitchell D.N. et al, 1978]. This finding was complicated by the later discovery of mycoplasma contamination in some of the original isolates and the possibility was raised that the isolates were the result of adventitious contamination during the isolation procedures [Mitchell D.N. et al, 1979]. Later on, SV5 was detected by immunofluorescence technique in bone marrows from 60 % of
patients with MS but also in 30% of controls and thus it was concluded that SV5 was not a contaminant in the original report [Goswami K.K. et al, 1983 and 1984]. Interestingly, immunofluorescence studies indicated that cross-reacting antigens between an antigen present in rat myelin and the major surface glycoprotein of SV5 may exist [Rastogi S.C. et al, 1979] [Goswami K.K. et al, 1985]. In the next series of experiments, Goswami et al showed that some MS' CSFs appeared to contain a significant proportion of oligoclonal antibodies against SV5 [Goswami K.K. et al, 1987]. To do so, CSFs from MS patients were incubated with purified SV5 or with purified MV and then centrifuged to pellet possible immune complexes. CSF supernatants were then compared by isoelectrofocusing before and after viral absorption. In CSFs of some MS patients, the majority of the oligoclonal pattern disappeared after viral absorption. This was not observed with MV or with the CSFs of the neurological controls tested. It was therefore concluded that, in some MS patients, most of the oligoclonal response was directed against SV5 [Goswami K.K.A. et al, 1987].

Conclusions on how viruses could be responsible for MS.

There are numerous proposed ways in which a virus could induce demyelination and some of these mechanisms have been elucidated by the study of viral induced demyelination in animals (see the corresponding sections).

Direct effect of the virus.

A virus can be cytopathic to oligodendrocytes and therefore induce demyelination by death of the myelin-producing and maintaining cell or by interfering with the normal metabolism of the myelin-producing cell (as it appears to be the case in progressive multifocal leukoencephalopathy). In this case a secondary inflammatory reaction may occur in response to this destruction as a scavenger effect.
Bystander effect due to viral infection.

On the other hand the inflammatory reaction may be responsible for the demyelination when an immunological response is triggered against viral antigens expressed during a persistent infection (as possibly in the case of Theiler's virus encephalitis). The destruction of the oligodendrocytes is, in this case, a bystander effect of the immune response to viral infected oligodendrocytes/myelin. This destruction could occur and be perpetuated non specifically for example by macrophages [Cuzner M.L. et al, 1988]. Antigenic modulation at the level of oligodendrocytes due to an antibody response against some viral antigens could induce a persistent infection without an overt inflammatory reaction and changes in antiviral titers influence the relapse in case of relapsing remitting demyelinating disease [Fujinami R.S. et al, 1984] [Randall R.E.R. et al, 1990].

Bystander effects can be promoted by soluble factors released during a normal anti-viral immune reaction and these soluble factors e.g cytokines released by lymphocytes and macrophages or enzymes released by macrophages could have a deleterious effect on the myelin. Such soluble factors toxic to myelin have already been described e.g complement [Scolding N.J. et al, 1989], proteinase from disrupted myelin sheaths [Chantry A. et al, 1988] or cytokines secreted by lymphocytes [Owen S.J. et al, 1988].

Autoimmunity as a bystander effect of viral infection.

The other possibility is that the demyelination is due to an autoimmune response triggered by a viral infection. In this case the virus need not be present in the brain. An autoimmune response is an immune reaction orchestrated by components of the immune system towards a component(s) of the body - e.g the myelin or the cell producing the myelin. The autoimmune response could be triggered by tissue damage due to viral infection (the viral damage is not the direct cause of demyelination but only a trigger).


This autoimmune reaction could for example be triggered by a release of sequestrated autoantigen normally hidden from the immune system e.g myelin basic protein (MBP) is normally not exposed in the mature myelin sheath in contrast to proteolipid protein (PLP) which is normally exposed [Norton W.T., 1984].
b. Presentation of autoantigen through increased expression of HLA molecules.

Along the same line of thought, it has been hypothesised that tissues threatened by viral infection increase their level of HLA molecule expression through the action of interferon or other cytokines [Dalgleish A.G. et al, 1987]. This facilitates presentation of viral peptides but may inadvertently raise presentation of tissue specific peptides (MBP or other brain specific antigens) to levels that activate previously quiescent autoreactive T cells [Parham P, 1991]. This hypothesis follows on a more general hypothesis for tissue specific autoimmunity, according to which T cells directed against tissue-specific peptides presented by aberrantly expressed HLA molecules are the cause of autoimmune disease [Bottazo G.F. et al, 1983 and 1986] [Hanafusa T et al, 1983] [Londei M. et al, 1984]. Autoreactive T cells have been demonstrated in normal people e.g against MBP [Martin R. et al, 1990] or against acetylcholine receptor [Sommer N. et al, 1991] and it is thought that they escape the normal negative selection (deletion) because these antigens may not be presented, in the thymus, during foetal life.

c. Insertion of a viral component into a host component.

Retroviruses produce latent infection in infected cells. Insertion of the viral genome into the genome of the cell is mostly random. The possibility that only part of the viral genome insert into the genome of the cell exists. This could induce transcription of a single viral protein during transcription of cellular proteins and expression of the viral protein alongside the cellular proteins. Some of the superantigens (see below) already characterised e.g the Mls system may have arise from this mechanisms. Such viral proteins could induce an autoimmune reaction if not tolerised.

d. The 'superantigen' hypothesis.

Superantigens induce proliferation, deletion or anergy of certain T cell subsets through interaction of the superantigen with specific Vβ segments of the corresponding T cell receptors [Acha-Orbea H. et al, 1991]. It has been hypothesised that among the potential T lymphocytes stimulated, there may be lymphocytes specific for myelin component especially MBP [Rudge P., 1991].
e. Incorporation of a host component into a viral component.

Another interesting possibility is the triggering of an autoimmune reaction by the incorporation of host antigens into viral envelopes during viral release by 'budding' i.e any virus with a viral envelope e.g retroviruses or paramyxoviruses. This incorporation of cellular components e.g glycolipids into viruses has been proposed as a possible cause of natural tolerance breaking leading to autoimmune reaction. Viruses could then act as helper-carrier for the glycolipids which are considered as haptens [Webb H.E. et al, 1984] [Dalgleish A.G. et al, 1987].

f. 'Molecular mimicry'.

The autoimmune reaction could also arise by 'molecular mimicry' - in other words as a result of molecular structures shared by host components and viral gene products [Fujinami R.C., 1988] [Srinivasappa J. et al, 1986]. In this case, the infectious agent does not need to be present in the brain or even in the body when neurological symptoms occur. Indeed, an autoimmune reaction due to molecular mimicry by an initial viral infection could be kept under control by suppression mechanisms orchestrated by the immune system ('tolerated'). The breaking of tolerance could then happen by a non specific phenomenon for example another viral infection. Thus, Sibley and colleagues have shown that MS patients have more viral infections before their attacks than a control population [Sibley W.A. et al, 1985]. So far, molecular mimicry between viruses and host components has been demonstrated mainly with intermediate filaments (intermediate filaments are a large part of the host structure). For example, Fujinami and colleagues have demonstrated cross-reactivity with different antibodies between herpes and measles virus proteins and human intermediate filaments [Fujinami R.S. et al, 1983] [Dales S. et al, 1983] [Nigg E.A. et al, 1982].

Fujinami and Oldstone immunised rabbits with a peptide of hepatitis B virus polymerase which shares some homology with an encephalitogenic site of myelin basic protein. Consequently, the rabbits developed neuropathological signs resembling experimental allergic encephalomyelitis; an antibody response and a T cell proliferation were demonstrated against both the myelin and the hepatitis B virus polymerase [Fujinami R.S. et al, 1985].
Molecular mimicry can be determined by computer analysis of the primary sequence of the proteins of interest [Jahnke U. et al, 1985] [Shaw S-Yet al, 1986] [Weise M.J. et al, 1988] [Souberbielle et al, 1991]. However, it is likely that proteins with different primary structures could also induce molecular mimicry if their three dimensional structure are similar as Rudensky and colleagues eluted proteins from a mice MHC molecule and found different peptide sequence bound to this MHC molecule [Rudensky A.Y. et al, 1991]. In addition flanking regions of an antigenic site may influence its presentation [Eisenhhor L.C. et al, 1992] and thus flanking regions may be an additional important factor in molecular mimicry.

Some have also proposed that 'molecular mimicry' could arise between host antigens and idiotypes of antibodies specific for viral antigens. Idiotypes are antigens of the variable regions of antibodies and it is thought that some of these idiotypes are the internal image of the antigen specific for the antibody. It has been hypothesised that the immune system is thus possibly regulated through this network of idiotypes [Jerne N.K., 1974]. Thus a response against an idiotype (anti-idiotype) could react against the antigen [Plotz P.H., 1983]. For example it has been demonstrated that a monoclonal antibody raised against an antibody specific for the reovirus 3 haemaglutinin reacts also with normal lymphoid and other normal cells [Nepom J.T. et al, 1982]. Cooke and colleagues have proposed that T cell specific for idiotype on antibodies specific for an environmental antigen could recognise an autoantigen through molecular mimicry between the idiotype and the autoantigen (T- inducer bypass) [Roitt I.M., 1984] and it is one of the theories which deals with the disruption of the complex regulation of the immune system (see below).

It has also been hypothesised that autoimmunity could arise through non specific polyclonal activation of B cells after viral infections. Some of the antibodies produced could be directed to autoantigens. The classic example given is Epstein Barr virus infection which induce polyclonal activation of B cells [Roitt I.M., 1984].

g. Disruption of the immune system regulation.

The immune system appears to be a closely regulated network of effector and suppressor cells and it has been proposed that autoimmunity results from a disruption of this regulatory system [Hafler D.A. et al, 1991]. For example, destruction of a subtype of...
lymphocyte (e.g. some specific suppressor cells) after viral infection could in turn trigger an autoimmune response towards a host component. On the other hand, stimulation of autoaggressive cells could also follow viral infection through non-specific activation of certain T cells or stimulation of T cell at a paracrine level. Brod and colleagues demonstrated that activated T cells expressing high surface density of adhesion molecules were able to trigger neighbouring resting T cells to proliferate via these adhesion pathways. In the same way, this non-specific T cells stimulation could arise thus after viral infection [Brod S.A. et al, 1990].

In the context of MS, all the possibilities developed above concerning infections of the myelin and/or the oligodendrocyte (or autoimmune reaction towards one of these biological structures) as a system could, in theory, also be applied to the brain vessels.

THE CEREBROSPINAL FLUID (CSF) AND MULTIPLE SCLEROSIS.

The CSF is of importance in multiple sclerosis for 2 reasons. The first is that oligoclonal intrathecal production of IgG can be demonstrated in over 90% of patients with definite MS. The second reason is that the CNS is bathed by the CSF and thus the CSF may reflect more closely than the blood the changes that occur in the CNS in MS patients.

General considerations.

a) Circulation of the CSF.

The CSF is derived from the plasma and 70% is produced at the blood-CSF barrier of the choroid plexus. The remaining 30% is derived from the capillary bed and meninges (18%) or from metabolic water production (12%) [Leibowitz S. et al, 1983]. The CSF has a volume of about 140 ml and is secreted at the rate of 0.35 ml/min, which means that the entire volume is replaced 3 to 4 times a day.

The CSF leaves the ventricular system and enters the cisterna of the subarachnoidal space which envelopes the spinal and the
cortical regions of the brain. The CSF then leaves the subarachnoidal spaces through the arachnoid villi into the venous system (The CSF is secreted by the arterial system and leaves through the venous system). With increasing distance from the ventricles there is an increasing concentration of the total protein between blood and CSF via the BBB and extracellular fluid. In summary, the transport systems between the brain compartments are multiple: one goes from the blood to the extracellular fluid (ECF) of the brain and can go back to the blood. Alternatively, the transport could be blood-brain-ventricle-CSF or blood-brain-subarachnoidal spaces-CSF. Another transport system is blood-meningeal vessels and then CSF. Additionally, CSF can be moved around via the lymphatics of the head and neck [Esiri M.M. et al, 1990].

Additionally, the local production of proteins from the cells in the brain has to be considered as a source of increased protein concentration in extracellular fluid and subsequently in CSF.

b) The composition of the CSF in patients with MS and in normals.

The CSF in MS patients as in the normal situation is crystal clear and of normal pressure [Walsh M.J. et al, 1983]. The total leukocyte count is normally (< 5 leukocytes per mm³). In 60% of MS patients, it is <5, in 95% of all MS patients <15 and <20 in 99.7% and rarely exceeds 50. The average leukocytes count in all MS patients (in remission and in relapse) is 5.6. As the duration of MS increases, the total leukocytes count becomes more normal [Walsh M.J. et al, 1983]. The differential distribution of CSF leukocytes is normal in MS patients [Tourtellotte W.W., 1970][Walsh M.J. et al, 1983]. As an example, the average count in 20 normal individuals and 106 patients with multiple sclerosis measured by Tourtelotte is presented per ml (Normal/MS patients respectively): large lymphocytes 900/2520, small lymphocytes 900/3000, monocytes 120/300, polymorphonuclear 10/12, eosinophils 2/6, plasma cells 3/24. Although some studies have reported a correlation between pleocytosis and presence of a relapse of clinical activity, this was not observed by other neurological studies [Walsh M.J. et al, 1983][Tourtellotte W.W.C. 1970].
The total protein concentration in normal CSF is 55 mg/dl. The total protein in MS CSF is < 55 mg/dl in 77% of patients, < 89 in 95% and <108 in 99.7%. In the same way, the CSF albumin concentration is normal (<32 mg/dl) in 77% of MS patients, < 53 mg/dl in 95% of patients and <65 mg/dl in 99.7% of patients.

The concentration of IgG in the CSF is normally 10-40 mg/liter. In about 85% of MS patients, the IgG/total protein is abnormal > 0.15. The IgGs are intrathecally synthesized and the commonly used index for measuring intra-BBB IgG synthesis is the Link/Tibbling IgG index: (IgG CSF/IgG S) divided by (Alb CSF/Alb S); a value exceeding 0.7 is considered abnormal and a sign of intra-BBB IgG synthesis. In about 90% of MS patients this value is abnormal [Link H. et al, 1977] [Hershey L.A. et al, 1980].

The rate of synthesis of the IgG per day has been evaluated by radioisotope methods (see [Walsh M.J. et al, 1983]). The average production was 29 mg/day in 127 patients suffering from MS. It was higher in the more acute cases and tended to increase with age, duration of disease, number of relapses and with elevated CSF leukocyte counts. The IgG production decreased with ACTH or prednisone treatment [Tourtelotte W.W. et al, 1978].

The interesting feature of the CSF of MS patients is the presence of an oligoclonal pattern of the immunoglobulins G.

**Oligoclonal bands and MS.**

a) Definition and description.

The IgGs, in more than 90% of patients with definite MS, show a qualitative abnormality as demonstrated by electrophoretic techniques [Lowental A.M et al, 1960]. The so called oligoclonal bands are of restricted heterogeneity and the banding resembles that found in a serum after repeated immunization [Osterland C.K. et al, 1966]. The oligoclonal bands are cathodic in location [Siden A., 1980] and means that the isoelectric point (Pi) of these oligoclonal ranges from 6 to 9 in general. The banding pattern is unique for each MS patient and it is remarkably constant even if patients are studied over 5 or 10 years or are in relapse [Hershey L.A. et al, 1980] [Livrea P. et al, 1981]. A small number of MS patients appear never to develop oligoclonal banding throughout the course of their diseases despite having autopsy-proved disease: this correlates with the relative or absolute absence of plasma cells in plaques [Ebers
G.C. et al, 1983]. The oligoclonal bands are known to be IgG. IgG oligoclonal bands are reduced but not eradicated by ACTH and/or steroids but the same pattern remains. This diminution is correlated with the decrease of IgG production (see above).

Mattson and colleagues eluted the IgGs of different plaques from the brain of a MS patient after autopsy and also from the brain of a patient who had died from subacute sclerosing panencephalitis (SSPE). They observed that unlike SSPE the pattern of oligoclonal banding produced was different between plaques although some of the oligoclonal bands were shared between different plaques. They concluded that the production of oligoclonal IgG may be due to the non-specific stimulation of B cells trapped in the CNS (leading to this heterogeneity of oligoclonal pattern between plaques) but that the oligoclonal bands shared between plaques may be specific for an antigen critical for the pathogenesis of MS [Mattson D.H. et al, 1980].

In addition to this restricted heterogeneity, in the CSF of MS patients there is an altered kappa/lambda light chain ratio (about 50 % of MS patients have a predominance of light chains of the kappa type which cannot be demonstrated in their serum) [Link H. et al, 1970][DeCarli C. 1987] and a relative IgG subclass restriction with predominance of IgG 1 subclass [Vandvik B. et al, 1976].

b) Other immunoglobulins in the CSF.

IgM immunoglobulins are elevated in the CSF of 40-50 % of multiple sclerosis patients [Williams A.C. et al, 1978]. Chiodi and colleagues have showed that in some MS patients a small proportion of these IgMs is directed against viral proteins (mumps, measles or varicella) [Chiodi F et al, 1987]. IgM is usually not visualised in polyacrylamide gels because of restrictive pore size for large molecules. A discrete band corresponding to IgMs is sometimes seen in MS CSF on agarose-gel electrophoresis.

IgAs are elevated in a small proportion of patients with MS (<10 %). It does not appear however that IgAs show an oligoclonal pattern [Link H. et al, 1979]. Very little is known about the other classes of immunoglobulins.
c) Other diseases show CSF oligoclonal pattern.

Other diseases show an increase of intra-BBB IgG synthesis and also oligoclonal banding. Ebers and colleagues classified these conditions into 2 groups. The first consists of a variety of disorders in which there is a local immune response in the nervous system in the CNS. These disorders include meningitis, encephalitis, paraneoplastic syndromes, neurosyphilis, polyneuropathy. The second group consists of a variety of disorders in which there is no obvious cause for oligoclonal bands. The most common of these disorders is stroke and oligoclonal banding can also been found in association with psychosis, headache, brain tumors etc, [Ebers G.C., 1984]. More recently, oligoclonal immunoglobulins have been described in the CSFs of patients suffering from tropical spastic paraparesis and 'AIDS dementia complex' (see chapter on viruses and multiple sclerosis)

d) Techniques used to visualise oligoclonal bands.

Oligoclonal bands can be detected by electrophoresis and isoelectric focusing. At first CSF had to be concentrated for visualisation. The best method which does not appear to lead to a drastic loss of CSF proteins is by the use of the collodion bag method of Kaplan and Johnstone [Kaplan A. et al, 1966]. With the use of silver stains or immunological methods, the CSF oligoclonal banding can now be visualised with unconcentrated CSF.

Three methods for visualisation of oligoclonal bands are described in this study: isoelectric focusing (IEF) techniques on polyacrylamide gel combined with silver staining, the immunoprinting method (IPP) and the immunofixation method (IF). Only the last method requires concentration of the CSF.

e) Specificity of the oligoclonal IgG in MS.

A very small proportion of the oligoclonal IgGs is specific for viral proteins (see section on viruses and MS). However, the specificity of most of the oligoclonal IgGs is unknown. It is thought that at least some part of this oligoclonal response is due to non specific stimulation of plasma cells trapped in the CNS. However,
some researchers still feel that a relevant specificity is present somewhere in the oligoclonal response so commonly present in MS.

Other components studied in the CSF in relation to MS.

In the CSF of MS patients, Morgan and his colleagues have described the significant consumption of the terminal component of complement (C9) [Morgan B.P. et al, 1984]. They also described the presence of membrane attack complexes of complement in the CSF of some MS patients [Scolding N.J. et al, 1989]. However, some other groups have not repeated the finding related to consumption of C9 finding only minor consumption of C9 [Halawa I. et al, 1990].

Other immunological parameters have been detected in the CSF of MS patients but their relevance to the MS process is still unknown. Schuller and colleagues detected the presence of anti-DNA and anti-RNA in about a third of the patients tested [Schuller E. et al, 1978]. Others have detected antismooth muscle antibodies in a very small proportion of patients with MS [Vandvik B. et al, 1977]. Anti myelin protein antibodies can be detected in the CSF of some MS patients but it may be that these antibodies are the sign of myelin breakdown or at most only partly responsible for this breakdown [Baig S. et al, 1991][Ichikawa T. et al, 1988].

More recently Zanetta and colleagues observed that 47 out of 51 patients with multiple sclerosis had antibodies specific to a mannose-binding protein, the cerebellar soluble lectin (CSL). Only 30 out of 188 neurological controls showed a positive reaction and 14 out of the 30 controls positive for anti-CSL were over 60 year of age, an age-group not representative of multiple sclerosis [Zanetta J.P. et al, 1990]. This observation needs to be repeated to evaluate the potential diagnostic, but also aetiopathogenic relevance of CSL in MS.
IMMUNOLOGY OF MULTIPLE SCLEROSIS.

Evidence of immunological involvement in MS is given by the histopathology of the lesions (see section on the histopathology of MS). Moreover, there are signs that the immune system is perturbed as for example the occurrence of oligoclonal IgGs in the CSF of MS patients (see section on CSF and MS) and quantitative and functional changes have been described in the peripheral system. Epidemiologically MS is associated with some alleles of the HLA class II (DQw1 in the north east of Scotland). Class II genes are also called immune response genes as they influence the immunological response to each individual making an individual's immune response unique in relation to all antigens that she or he may encounter. Association with certain HLA alleles is found with all the diseases which are thought to be autoimmune [Roitt I., 1984]. In addition, immunosuppressive agents such as corticosteroids [Milligan N.M. et al, 1987] can diminish the clinical symptoms of the disease although they are inefficient upon the long term course of the disease. It is however not evident that all the immunological features seen in the disease are related to the aetiopathogenesis of the disease or if they are mainly consequences of the MS lesions.

One important theory of MS is that the disease is autoimmune in origin perhaps triggered by a viral infection. The autoimmune theory is supported by experimental allergic encephalomyelitis (EAE), an induced animal disease considered a model for MS. EAE is induced in genetically specific animals by the injection of myelin basic protein (MBP) or proteolipid protein (PLP) (both proteins of the myelin) with Freund's adjuvant. As in MS, the importance of the BBB has been recognised in EAE (see below). A great deal of work has been done with MBP in humans but the evidence to implicate this protein in the etiopathogenesis of MS is slim. In this chapter, a brief review on the current knowledge and theory of the immunology of MS will be presented.
Peripheral blood and CSF immune abnormalities in MS.

The rational behind studying the immune system in MS is to understand the cellular and soluble factors that may initiate, mediate but also regulate the disease process. The autoantigen(s) is not known in MS and therefore the immunological studies in MS are limited to non antigen-specific assays.

A number of immune abnormalities have been observed in the peripheral blood of patients with MS. However, due to the difficulty in longitudinal studies, some of the findings are still questioned. Moreover, it appears that these abnormalities tend to be more pronounced in chronic progressive MS, and, from the literature, it is sometimes difficult to know what type of disease has been included in a study. The same abnormalities may happen in relapsing patients but studies of this group are complicated by imprecision in judging disease activity by clinical criteria alone. Moreover the correlation between clinical manifestations of the disease and the underlying pathological activity are poor. Using NMR, many silent lesions can be identified that often do not correspond with any clinical signs [Ormerod I.E.C. et al, 1987][Oger J. et al, 1987].

Some of the cellular abnormalities are interpreted as a decrease in suppression and immunoregulation [Hafler D.A. et al, 1989][Freedman M.S. et al, 1988]. In the peripheral blood, there is a decreased mitogen or autologous mixed lymphocyte reaction (AMLR) in patients with progressive disease [Antel J.P. et al, 1978][Hafler D.A. et al, 1985][Hirsch R.L., 1986][Baxevanis C.N. et al, 1987][Chofflon M et al, 1988]. A reduced suppression activity has also been correlated with formation of new lesions visualised by NMR in relapsing-remitting patients [Oger J. et al, 1987]. The suppression-induction function has been associated with a subset of CD4+ T cells harbouring CD45R+ molecules (recognised e.g by the 2H4 mAb) whereas the helper-inducer function has been associated with a mutually exclusive CD4+ subset harbouring CD 29+ molecules (recognised e.g by the 4B4 mAb). On the other hand the suppressor-effector function is attributed to the CD8+ population although reports of CD4+ and non-T cell suppressors exist [Freedman M.S. et al, 1988]. In progressive and in relapsing-remitting disease, a decrease of the CD4+CD45+ subset (suppressor-
inducer) has been observed [Rose L.M. et al, 1988] [Morimoto C. et al, 1987].

Other cellular abnormalities may be related to the consequence of the decrease in suppression and account for a state of T-cell-activation. Peripheral T cells from relapsing and progressive patients express 'activation' antigens (e.g CDw26, VLA-1, IL2 receptor) when compared with non-MS controls [Noronha A. et al, 1980] [Hafler D.A. et al, 1985 b and c] [Kerman R.H. et al, 1988].

In the CSF, similar changes can be detected. There is an over-representation of CD4+ cells compared to the blood [Cashman N. et al, 1982]. There is also an increased number of CD4+ CDw29+ and decreased number of CD4+ CD45R+ T cells [Chofflon M.M. et al, 1989]. The number of activated T cells is also high [Noronha A. et al, 1980] [Bellamy A.S. et al, 1985]. In progressive patients, it appears that the T cells are oligoclonal in nature. Moreover, oligoclonal T cells are also present in the blood and these oligoclonal T cells share the same T cell receptor Vb usage with their counterpart in the CSF. However, it appears that each MS patient is unique as TCR Vb usage of the oligoclonal T cells is not identical between patients [Lee S.J. et al, 1991].

In the brain, the changes are again similar with an increased number of CD4+ CDw29+ and decreased number of CD4+ CD45+ T cells [Sobel R.A. et al, 1988], increased activated T cells (IL2 receptor positive) and activated macrophages [Traugot U. et al, 1983] and presence of Class II on astrocytes [Hofman F.M. et al, 1986]. All the ingredients to fuel an inflammatory immune response are present. Moreover cytokines are detected in plaque areas [Hofman F.M. et al, 1986] for example TNF [Hofman F.M. et al, 1989] [Selmay K. et al, 1991]. The consensus on CD8+ T cells is that they tend to predominate at the edge of the plaques [Sobel R.A. et al, 1988]. However it is not clear whether these CD8+ T cells are cytotoxic T cells expanding the edges of the plaques or if they are suppressor T cells preventing the centrifugal expansion of the plaque.

Along with the cellular changes detected in the MS brain, there is the characteristic production of oligoclonal IgG, the significance of which is still unknown (see the CSF section).
Studies on a possible autoantigen in MS.

a) Antigens of the myelin.

The molecular structure of myelin has been studied extensively during the last decades. Lipids constitute 70-80% of the mass of the myelin. The distinguishing feature of myelin lipids is the high content of the galactosphingolipids, cerebroside and sulfatide [Norton W.T., 1984]. Glycosphingolipids are known to be lipid haptens and specific antibodies can also be raised against cerebrosides and sulfatide. It appears that myelin cerebroside is a specific marker of CNS oligodendrocytes [Raff M.C. et al, 1978].

The major proteins of myelin are myelin basic protein (MBP) (in human myelin, there are at least 2 basic proteins, a 18,500 kd and a 21,500 kd proteins), proteolipid protein (PLP) (25 kd) and the Wolfgram proteins group (between 42,000 and 50,000 kd) [Cruz T.F. et al, 1985]. The lower molecular weight protein of this group has been shown to be identical to the myelin 2'3' cyclic nucleotide 3' phosphodiesterase [Drumond R.J. et al, 1980]. Another protein of myelin is myelin associated glycoprotein (MAG) of molecular weight 100,000 kd for the native glycosylated protein [Slazer J.L.et al, 1987]. Other proteins of myelin are known to have enzymatic activity and at least 20 enzymes have been described in myelin; some appear to be myelin specific.

b) T cell autoantigens.

Many attempts have been made to define the primary target(s) within the CNS for a possible autoimmune reaction. Attention has been focussed on MBP since EAE can be induced by MBP. Moreover as MBP-specific T cell clones are sufficient to transfer EAE in rodents and because of the T cell predominance in MS lesions, the T cell response against different myelin antigens has been extensively studied in MS. Also, as it is known that the T cell immune response is 'restricted' by MHC molecules and because there is a genetic association with some HLA class II alleles and MS (particularly HLA DR2 and HLA DQw1), it is thought that the T cell arm of the immune response is central in the aetiopathogenesis of MS.
The type of assays for assessing the T cell immune response are limited for technical and ethical reasons. For example, skin testing (a good indicator of the delayed type hypersensitivity (DTH) \textit{in vivo}) is unethical with myelin antigens in MS patients.

Peripheral blood mononuclear cells (PBMC) from patients with MS at various stages of the disease and from control subjects have been studied for reactivity to CNS extracts and to MBP [Lisak R.P.B. et al, 1974, 1978 and 1984] [Rocklin R.E. et al, 1971] [Hughes R.I. et al, 1979] [Colby-Germinaro S.P. et al, 1977 a and b] [Johnson D. et al, 1986]. However, most studies have been done using MBP and not with myelin extracts. Indeed, in the literature there are very few studies which have looked at the T cell response against whole myelin [Rocklin R.E. et al, 1971] [Lisak R.P.B. et al, 1978] [Brockman R.W et a, 1970] [Barfeld H. et al, 1970] and those that have been done were carried out 20 years ago when the T cell proliferation assay (or lymphoblastic assay) protocol (the proportion of lymphoblasts was counted on a slide after incubation with the antigen) was different from the usual protocol nowadays (the incorporation of $^3$H thymidine in PBMC is counted after incubation with antigen). From all these studies, no conclusive T cell-antigen (s) has been found. Some patients show a definite T cell reactivity against some myelin antigens e.g MBP, but it is not clear whether this immune response induces the myelin breakdown or whether it is secondary to it. Moreover the majority of studies described some reactivity in normals and neurological controls. T cells which are specific for MBP have been cloned from the peripheral blood lymphocytes and from the CSF of MS patients but T cell-clones with identical specificity have been obtained from normal or neurological controls [Burns J. et al, 1983 and 1986] and proliferation have been obtained in normal individuals with peripheral blood lymphocytes incubated with MBP, PLP or MAG [Johnson D. et al, 1986].

Other sources of lymphocytes apart from the peripheral blood and the CSF can be used to study the T cell reactivity in MS patients. For example, the study of cloned T lymphocytes from MS lesions or from local lymph nodes may be more rewarding. Due to logistic and technical problems, there is only one study published in the literature which reports the successful cloning of T cells from MS lesions. Hafler and his colleagues cloned T lymphocytes from 2 MS plaques of a post mortem brain from a 62 year old man who had MS for 34 years and who was in progressive phase of his disease when he died.
They had previously isolated autologous antigen presenting cells (for the T cell cloning procedure and the T cell proliferation assay) before the death of this patient (D. Hafler, personal communication). They derived 57 clones which were tested for their reactivity to MBP and PLP. None of the clones proliferated with MBP or PLP although these T cell clones proliferated with the mitogen PHA [Hafler D.A. et al, 1987].

From the above discussion it is clear that the source of the T cell for T cell reactivity studies may be critical and other studies similar to the one Hafler and his colleagues have performed are needed. Indeed, the origin of the possible autoreactive T cells has been debated [Hafler D.A. et al, 1989][Calder V. et al, 1989] since it is not clear if they are initially activated in the blood and then cross the blood-brain barrier or if they originate within the brain.

c) Fine specificity of MBP- specific T cells in patients with MS.

It has been recognised that specific T cell sites of MBP are responsible for the induction of EAE in animal. These T-cell sites correspond to short peptides and they are different in genetically distinct animals [Alvord E.C., 1985]. In human, the same restricted antigenicity is observed with MBP. Despite the lack of evidence, the major immunological MS research groups in the world are working on the premise that MBP is one of the autoantigens of MS. Thus the T cell sites of MBP have been defined fairly extensively [Martin R. et al, 1991]. Indeed a MBP peptide (MBP85-105) appears to be worth considering since it has been shown that this peptide is recognised by cytotoxic T cells or T helper cells restricted by Class II DR alleles associated with multiple sclerosis [Richert J. et al, 1989] [Ota et al, 1990]. Shared T cell receptor Vβ usage (Vβ 17) to this immunodominant region of myelin basic protein was demonstrated among individuals with specific T cells to this peptide [Wucherpfennig K.W. et al, 1990]. This peptide shares homology with a number of viral proteins especially a protein P3-A from Coxsackie virus [Souberbielle B.E. et al, 1991]
d) B cell autoantigens.

The studies performed on MS patients in relation to a possible antibody response to myelin components are similarly inconclusive. There is no evidence that pathogenic antibodies directed against a component of the brain are prevalent in MS patients. Brain extracts, oligodendrocytes, myelin lipids and glycolipids as well as myelin proteins have been tested in different humoral assays but to no avail [Lisak R.P. et al, 1984]. Inconclusive results have been published on the level of antibodies to MBP found in the CSF of patients with MS. Some researchers were not able to detect such antibody [Chou C-H.J. et al, 1983] whereas others have detected low level antibodies to MBP which were part of the oligoclonal IgG production [Cruz M. et al, 1987].

There have been also reports of in vitro bioelectric blocking activity in the serum of patients with MS but the nature of the soluble factor involved in this process and its specificity has not been defined [Seil F.J. et al, 1976]. This bioelectric blocking factor(s) was claimed to block evoked electric activity in cerebral neocortex cultures. This factor(s) was never properly related to an antibody response and no further work was carried out on this matter.

Immune complexes have also been demonstrated in the CSF of some MS patients and some of the specificity of these immune complexes has been related to lipids [Lund G.A. et al, 1983], galactocerebroside, GM4 gangliosides and other brain antigens [Friedman J. et al, 1987].

e) Autoimmunity to brain vessels in MS.

Tsakuda and his colleagues have claimed that there is a humoral response against brain vessels in MS (the reader should refer to the BBB section of the introduction). No work have so far been done on a possible T cell response against the brain vessels in patients with MS and this is partly due to technical limitations. Undoubtedly, the brain vessels could play a role in the aetiopathogenesis of MS but the precise mechanism is still to be defined (see BBB section) and may not be related to an autoimmune reaction to the brain vessels. For example, it is interesting that whereas EAE can be induced by the subcutaneous injection of MBP-reactive T cells, injection of these cells directly into the brain does not induce the disease. Thus, this model suggests that T-cell
migration across the endothelium is critical for the initiation of an inflammatory reaction in the CNS [Hafler D.A. et al, 1989].

**Study of experimental autoimmune encephalomyelitis (EAE) and possible therapeutic intervention in MS.**

Finding the autoantigen(s) in MS is an important goal as it has important therapeutic implications. Indeed, different immunotherapeutic protocols have been explored with the animal model EAE where the autoantigen is known and well defined. Some of these immunotherapeutic protocols do prevent the disease and are based on the knowledge of the autoantigens (see below). Others show some promising activity and based on non-specific immunotherapy e.g. anti-TNF antibody can prevent EAE [Ruddle N.H. et al, 1990]. It is likely that a combination of specific (based upon the knowledge of the autoantigen) and non specific immunotherapies may be helpful in the treatment of MS which appears to be a more complex disease than the well defined disease EAE.

Different specific immunotherapies have been successfully tried in EAE. Animals have been vaccinated with inactivated autoreactive T cell clones or fragments of the T cell receptors derived from such clones. This approach has been claimed to be successful [Ben-nun A. et al, 1981] [Vanderbark A.A. et al, 1989] [Howell M.D. et al, 1989]. More recently, vaccination with TCR-derived peptide expressed on the T cells responsible for the disease has been shown to promote recovery from established EAE [Offner H. et al, 1991].

The use of specific monoclonal antibodies to critical determinants involved in the disease process has been another approach in order to prevent EAE. It has been shown that in H-2u mice, T helper cells recognising the peptide 1 to 9 of MBP are sufficient to induce EAE. These T helper cells share a common TCR Vβ gene segment - Vβ 8.2 . A monoclonal antibody specific for this subset of TCR injected into the animal prior to challenge with MBP has been shown to prevent the onset of the disease [Acha-Orbea H. et al, 1988] [Urban J.L. et al, 1988]. More recently Aharoni and colleagues were able to prevent the onset of EAE by injecting a monoclonal antibody specific for the MBP-Ia complex [Aharoni R. et al, 1991].
A third approach using specific immunotherapy for the treatment of EAE is based on the use of 'inhibiting peptides'. The peptide MBP1-9 is restricted by I-A\textsuperscript{u} in PL/J mice. Analogues of MBP1-9 have been developed which bind to I-A\textsuperscript{u} more effectively than the native MBP1-9. Coimmunisation with the analogue peptides at the time of disease induction significantly reduced the incidence of clinical disease [Wraith D.C. et al, 1989]. More recently, immunization with an encephalitogenic MBP peptide (1-11) with a single amino acid change at position 4 confers the capacity to prevent rather than induce EAE. A similar mechanism may account for the prevention or reduction in severity of EAE and chronic EAE after oral immunisation with myelin in animals. Another explanation is that oral immunisation can induce active suppression [Stephen H. et al, 1990].
CONCLUDING NOTES ON MULTIPLE SCLEROSIS.

In conclusion, multiple sclerosis is a difficult disease to diagnose as there is still no definitive laboratory or other test. Due to its relapsing-remitting course in most patients and due to the site of the disease, the brain, clinical research is difficult to undertake.

The epidemiology of the disease suggest that both environmental and genetic factors contribute to the disease process. The genetic predisposition seems to be polygenic with most of the associations described so far related to genes involved with the immune response. One theory suggests that an environmental factor(s) probably viral in nature induces, in genetically determined individuals, an immunological reaction which could be autoimmune in character.

Histopathological and immunological studies shows that there is a disregulation of the immune system in MS patients and that all the signs of a local, inflammatory reaction are present in the brain of MS patients. Moreover there is the production of the oligoclonal IgGs which are so characteristic of MS patients although not specific for this disease. The hallmark of the disease is the destruction of the myelin and thus a lot of effort has been spent in studying the possible immune response towards myelin antigens.

Since the lesions are perivascular, it is not unreasonable to suggest that the blood-brain barrier may be of some significance in the aetiopathogenesis of MS. However little data is available on this topic perhaps due to the difficulty in obtaining human brain endothelia and to the lack of an appropriate human brain endothelia cell line.
BACKGROUND TO THIS THESIS.

Studies over a number of years carried out at Mill Hill, London, and then at St Andrews (mostly by Dr. Kamal Goswami) have sought to ascertain if the paramyxovirus SV5 plays any role in the aetiology of some cases of MS.

These investigations were initiated after isolations of SV5 virus from MS bone marrows. Later on it was also shown that a third of the MS patients tested had antibodies specific for SV5 in their sera. The interest in SV5 and MS was further stimulated when it was observed that an epitope on the major surface glycoprotein of the virus cross reacted with an antigen present in myelin when tested with the immuno-fluorescence technique on cross sections of rat brain (see introduction on viruses and MS, the paramyxovirus section).

The next series of experiments (mostly done by Dr Goswami) analysed the oligoclonal antibodies present in the CSFs of MS patients on the basis that if SV5 played a role in the disease then it seemed likely that antibodies against the virus may compose a significant proportion of this oligoclonal response. The CSFs were screened by immunofluorescence techniques which indicated that 40-50% of the MS CSFs had antibodies to either SV5 or PF-2. The immunofluorescence technique cannot distinguish between PF-2 and SV5 because of cross reactions between these two viruses. Further characterisation of the CSF antibodies was made by using techniques which could distinguish between SV5 and PF-2 e.g by radioimmune precipitation (on the basis that the major surface glycoprotein, HN, of PF-2 and SV5 does not cross react [Goswami KK. et al, 1987]). This demonstrated that a significant proportion of the CSFs showing positive IF results contained antibodies against SV5. The most significant finding was that by using an immunoabsorption technique some of these CSFs had oligoclonal bands which were absorbed by preincubation with purified virus SV5 and even in one case with the purified virus SV5 HN glycoprotein. Moreover, I searched possible homologies between the known SV5 proteins and the National Biomedical Research Foundation protein sequence database using the FASTA program [Pearson W. et al, 1988] in the University of Wisconsin Genetics computer group package [Devereux J. et al, 1984] maintained at the Daresbury laboratory and also used the PSQ program from this package to compare the known myelin proteins.
and the SV5 proteins. A high score was recorded when the SV5 HN protein was compared to myelin associated glycoprotein (MAG) [Salzer J.L. et al, 1987] corresponding to a 20 amino acids stretch. The homology between MAG and SV5 HN is shown in figure IntroB-1.

**Figure IntroB-1:** Homology between myelin associated glycoprotein (MAG) and the HN protein of SV5. The solid lines represent identical amino acids between the 2 proteins and dashed lines represent structural homology between amino acids.

It was then decided that similar experiments should be repeated by independent researchers because of the potential significance for MS of these observations. Professor Erling Norrby and Dr Bodvar Vandvik (Norway) and Dr Brendan McLean (from the Institute of Neurology, Queen Square, London) were asked to carry out confirmatory tests. However, the techniques used in Norway and in London were different from the techniques in use at St Andrews.

The Scandinavian group used the 'imprint immunofixation' method which had been originally developed by them. The method depends on the ability of the oligoclonal bands displayed (after concentration of the CSF) by isoelectric focussing on polyacrylamide gels to be transferred sequentially to agarose gels containing different virus antigens. The antibodies which attach are then detected by 125 I labelled anti-human IgGs. The virus antigens are generally crude extracts of infected cells and controls are derived from extracts of uninfected cells (see Material and Methods and Results section). They studied 30 CSFs of MS patients and found that 7 had antibodies against SV5 and that these antibodies were
intrathecally synthetised (by comparison to the serum with the immunoprinting method). Most of these 7 patients showed intrathecal synthesis against other virus antigens. However, no correlation was observed between the patterns of the intrathecal SV5-IgG bands and the total IgG oligoclonal bands and it was concluded that SV5 intrathecal synthesis was not a major component of the total oligoclonal population. Further analyses also suggested that the bands noted were resulting from cross reacting antibodies to the other related paramyxoviruses PF-2 and mumps.

The second study was carried out by Dr B. McLean at St Andrews. The technique, immunoblotting, was used with CSFs brought from the London area. Unconcentrated CSFs were separated by isoelectric focusing on agarose gels and then blotted onto nitrocellulose coated with antigens. This method limited the possibility of denaturing the antigens but suffered from the lack of internal controls (see below). The basic results indicated that out of 14 MS CSFs, four were positive for the SV5 virus preparation, two of these showing an oligoclonal pattern. Three of the ten controls were also positive, one showing oligoclonal bands. On testing with purified SV5 HN only one of the MS showed a positive oligoclonal response whereas four of the controls did (one extra). Test with PF-2 showed equally diverse results, two of the MS CSFs and three controls reacting. Some MS and control CSFs also reacted well with Sendai virus (a mouse virus and presumed negative control). It was therefore a matter of contention whether or not these results could be explained by possible cross reactivity between related viruses e.g a possible cross reactivity between Sendai virus and PF-1 or PF-3 but another possibility was that the technique suffered from the lack of specificity.

The first aim of the work presented in this thesis was to test new CSFs of MS patients with the immunoabsorption technique. Secondly, results obtained by ourselves and the other 2 groups stimulated the need for an independent assessment of the 3 techniques used (imprint immuno-fixation, immunoblotting and immunoabsorption).

This study also relates to our supposition that viruses may play an aetiological role in the disease process. We therefore hypothesised that MS is an autoimmune disease induced by viral infection (see Introduction - Viruses and MS). It would therefore be critical to define autoantigen(s) concerned as a route to uncovering the nature
of the virus (or viruses) involved. Most of the studies carried out to date on the nature of the MS autoantigen have focussed on myelin, but more particularly on the study of only one protein, myelin basic protein (MBP) (see Introduction - Immunology of multiple sclerosis). However, the role played by the blood-brain barrier (see the blood-brain barrier section) or by other components of myelin in the aetiopathogenesis of MS have not been pursued to any great extent principally because of a limitation in the techniques available to study them. There is thus a need for a technique which will permit one to test the immunological response to many different antigens from the brain. Studies on the BBB have also been limited by the lack of a human brain endothelial cell line. It will also be apparent that other aspects of blood-brain permeability could gain from the availability of a human brain endothelial cell in culture e.g. very little is known about viral infections of the blood brain barrier.
MATERIAL AND METHODS.

MM1: POLYACRYLAMIDE ISOELECTRIC FOCUSING OF CSF.

Reference [Schipper H.I. et al, 1984].

Material
- Isoelectrofocusing (IEF) Ampholine PAG gel-plates (pI interval 3.5-9.5) (Pharmacia), stored at +4°C.
- IEF gel apparatus (LKB) with a cold water cooling system
- Power pack (LKB Multiphor II, electrophoresis unit) connected to the IEF apparatus.
- Paper applicators for the samples (LKB or Whatman)
  - Buffer applicator papers (LKB or Whatman).
- Anode buffer: 0.1 M H₂SO₄.
- Cathode buffer: 1 M NaOH.
- Very fine forceps.

Method.
- The water cooling system was switched on.
- Kerosene was applied over the apparatus flat bed.
- The IEF gel was cut to the desired dimensions and applied to the apparatus bed (bubbles were avoided).
- Anode, cathode blotting paper applicators and samples paper applicators were placed on the gel.
- Anode and cathode buffers were applied on their respective applicators.
- 10 μl of each test sample was applied on its applicator.
- The gel was run at a constant current of 35 mA until it reached 1000 volts and then set at a constant voltage of 1000 volts for 105 minutes. (35 mA was adequate for the entire gel of 20 cm long; half a gel was run at 15 mA).
- The samples paper applicators were removed from the gel after 20 minutes of electrophoresis (the electrophoresis was switched off and on during this process).
MM2: SILVER STAINING OF IEF GEL OR SDS PAGE GELS.

a/ Material - silver staining kit from BIO RAD:
1. Oxidiser (x10 strength stock solution): 20 ml of the stock solution was mixed with 180 mls of deionized distilled water. The oxidiser working solution was prepared the day the staining was to be done.
2. Silver reagent (x10 strength solution): 20 mls of the stock solution was mixed with 180 mls of deionised distilled water prepared on the same day that staining was carried out.
3. Developer: the 32 g of the stock powder solution were dissolved in a litre of deionized distilled water (the solution could be kept for at least a month at room temperature).
- deionized distilled water (DDW)
- glass dishes (at least two) to contain the gel for stain.
- ampholytes removal solution A (ARS-A): 30% (v/v) methanol, 10% (v/v) trichloroacetic acid, 3.5 % (v/v) sulphosalicyclic acid.
- ampholytes removal solution B (ARS-B): 30% (v/v) methanol, 12% trichloroacetic acid.
- fixative A (F-A): 40% (v/v) methanol, 10% (v/v) acetic acid.
- fixative B (F-B): 10% (v/v) ethanol, 5% (v/v) acetic acid.
- storage solution (SS): 5% (v/v) acetic acid.
- 'rocker'

b/ Method: The duration of incubation with the different solutions depended on the width of the gel. All the experiments used 0.75 mm gels (IEF or SDS PAGE) and thus the duration quoted below was for gels of 0.5-1 mm width.
The method described is for IEF gels.
- The gel was transferred into deionized distilled water-cleaned glass dishes. Cleaned dishes were used for each step of the silver staining technique.
- The gel was incubated with the different solutions as follows.
  - ARS-A: 1 hour
- ARS-B: 2 hours (the solution was changed every 40 minutes).
* - F-B: 15 minutes incubation.
- F-B: 15 minutes incubation.
- Oxidiser: 5 minutes.
- DDW: 5 minutes.
- DDW: 5 minutes.
- DDW: 5 minutes.
- Silver reagent: 20 minutes.
- Developer: the gel was developed until the solution turned yellow or a 'smokey' precipitate appeared. The developer was poured off and fresh developer added. The gel was incubated in developer until optimum development was obtained (the developer was changed every 5 minutes or when it became cloudy).
- The reaction was stopped with SS
- The gel was photographed, or, stored in the SS solution, in a sealed bag.

The silver staining protocol of SDS-PAGE gels was different as it was not necessary to remove ampholytes before the silver staining. The protocol begins with a 30 minutes incubation in F-A solution and then is similar to the IEF gel silver staining from the asterisk step marked (*) (above).
MM3: IMMUNOPRECIPITATION OF CSF.

(see figure MM3-1 for an outline of the technique).

a/ Material: - polypropylene centrifuge tubes for TL 100 centrifuge (Beckman).
   - TL 100 ultracentrifuge (Beckman).
   - purified virus preparation.

b/ Method: - The virus suspension was adjusted to give a protein content of 1-5 mg/ml.
   - The CSF samples were centrifuged at 350,000 g for 2 hours in the Beckman TL-100 at + 4°C.
   - Each aliquot (95µl) of the CSF supernatant was incubated with 5 µl of the virus preparation (or with PBS for the negative control) and after mixing, left overnight at + 4°C for absorption.
   - the CSF-antigen mixtures were then centrifuged at 350,000 g for 2 hours.
   - About 70 µl of the supernatant was removed and then subjected to IEF and silver staining.
Figure MM3-1: Outline of the immunoprecipitation of CSF for characterisation of possible antigen specific oligoclonal bands.
MM4: IMMUNOBLOTTING METHOD ON AGAROSE GEL.

This technique has been described by Moyle S. and colleagues [Moyle S. et al, 1984].

a/ Material:

1. Casting of the gel:
   - casting frame (247mm x 13.6 mm x 3 mm) (see figure MM4-1).
   - glass plate and bull-dog clips.
   - IEF agarose (Pharmacia)
   - glycerol.
   - D-sorbitol.
   - thermometer.
   - ampholytes solutions (Pharmacia), kept at + 4°C
     1. PI 3-10
     2. PI 8-10.5
   - Gel- Bond film (agarose gel support medium), ICN Biomedical Ltd, thickness 0.1 mm, width 127 mm.
   - syringe, needles and swabs.
   - 10 ml pipettes.
   - level spirit.

2. Isoelectrophoresis
   - isoelectrofocusing system (see acrylamide IEF section).
   - sample applicator (LKB or Pharmacia).
   - cathode and anode buffer (see acrylamide IEF).
   - tissue paper
   - glass plate + weight
   - blotting paper ( Whatman 3 MM)
   - nitrocellulose paper (Sartorius Gmbh D3400 Gottingen, Germany, Nitro-cellulose 200x200 ref: 054217; 0.2 μm pore size).

3. Immunoblotting visualisation
   - Blocking solution: 1 % (w/v) bovine serum albumin (BSA), 1 % (w/v) gelatin in PBS.
   - 'First antibody' for example for human CSF: [goat] anti-human IgG Fc receptor specific (Atlantic Ab).
   - 'Second antibody' : [Rabbit] anti-goat total immunoglobulins peroxidase linked (Dako)
   - Tween 20
   - 3-amino-9-ethylcarbazole (Sigma)
- 0.02 M (H.Cl) acetate buffer, pH 5.1, freshly prepared.
- Hydrogen peroxide (H2O2), kept at + 4°C.
- 'rocker' at 37 °C.
- air dryer.

b/ Method.

1. Casting of the agarose gel:
- The gel was made by mixing 27 ml of 10 % (V/v) glycerol in deionised distilled water and 0.3 g of agarose IEF and 3.6 g of D-sorbitol.
The mixture was dissolved by boiling and then kept in closed glass bottle where the gel set.
- The glass plate with the Gel-Bond and the casting frame fixed to the glass plates by bull-dog clip were warmed and then placed on a horizontal surface. The horizontal level of the plate was checked using a level spirit.
- The gel was warmed to 70 °C and 2ml of ampholyte (3-10, PI) solution and 0.5 ml of (8-10.5) ampholyte solution were added with a syringe and a needle.
- The gel was casted inside the casting frame. A pipette was used to spread the gel evenly. The gel set quickly and was kept in a moist atmosphere for some days.

2. Isoelectrophoresis of the agarose gel: 2 methods are described which only differ in the type of NTP sheet used.
When the pattern of the CSF oligoclonal bands was required, a standard NTP sheet was used in order to transfer the CSF immunoglobulins. If the assay attempted to localise virus-specific- oligoclonal bands, then the NTP sheet was covered with the purified virus preparation. (See figure MM4-2 for the outline of the method)
- The apparati were identical as those for the polyacrylamide method but the method differed somewhat.
- The cooling water system was switched on
- The bed of the apparatus was swabbed with alcohol.
- The gel was placed on the bed apparatus and the edges of the gels were trimmed with a scalpel (about 0.5 cm).
- A sheet of dry nitrocellulose paper, long enough to cover the gel, was placed on the gel and then taken off quickly and kept in distilled water for further use.
- The cathode and anode applicators were placed and soaked in their respective buffers.
- The samples were applied to the gel with the help of the sample applicator.
- Tissue papers were placed on both side of the gel, outside the anode and cathode applicators.
- The gel was run at 1500V, 150 mA (power 20). At the beginning of the run, the voltage was about 500 V.
- The sample applicator was removed after 40 minutes and the electrophoresis was continued for another 50 minutes (total time 1 hour 30 minutes).
- When the run was completed, the nitrocellulose sheet that had been in distilled water was applied for 10 seconds to the gel and then discarded.
- A second nitrocellulose paper, soaked in distilled water was applied to the gel and then covered by 10 blotting papers (Whatman 3 MM).
* If specific- virus oligoclonal bands were sought, the NTP was covered with a purified preparation of the virus (see below).
- A glass plate was placed on the top of the blotting papers and a weight was placed on the glass plate.
- The incubation period was 20 minutes.

3. Immunoblotting visualisation:
- The NTP was blocked in blocking solution for 1 hour 30 minutes.
- The excess was washed away and the 'first antibody' was incubated for 1 hour with the NTP on a 'rocker' at 37 °C (for a 10x20 cm sheet, 50 µl of 'first antibody' in the minimum volume of 0.1 % (w/v) BSA-PBS.
- the NTP sheet was washed 3 times in PBS
- The 'second antibody' (peroxidase-linked) was incubated with the NTP in the same conditions as the 'first antibody'.
- The NTP was washed 4 times in 0.5 % (v/v) Tween 20-PBS and twice in distilled water.
- The reaction was visualised with:
  10 mg of 3-amino-9-ethylcarbazole diluted in 6 ml of methanol and mixed with 44 ml of 0.02 M acetate buffer. Just before the development, 50 µl of H2O2 were added. The reaction was stopped with distilled water.
- The NTP sheet was air dried (this improved the contrast of the bands).

4. *For antigen blotting e.g. a virus preparation, the NTP sheet was prepared as follow:
- The NTP sheet was wet in PBS and then soaked with antigen for 2 hours at room temperature or overnight at +4°C.
- 10 ng (10-80 ng) of proteins / cm² of NTP for unpurified antigen were required. The antigen preparation was diluted in the minimum volume of PBS.
- The NTP was incubated in blocking solution for 1 hour and 30 minutes.
- The excess of the blocking solution was washed away.
- The NTP was then applied to the agarose gel (see end of section 2.).

Figure MM4-1: Casting frame for the preparation of the agarose gel for the CSF immunoblotting method.
Figure MM4-2: Outline of the immunoblotting method for characterisation of CSF oligoclonal bands.
MM5: IMPRINT IMMUNO-FIXATION (IIF) METHOD.

The IIF method has been described by H.J. Nordal and colleagues [Nordal H.J. et al, 1978] as well as the preparation of virus antigens for the IIF [Vartdal F et al, 1979].

a/ Material:- all materials used in the polyacrylamide isoelectric focusing
- barbital buffer: 86 ml of 0.1 M sodium barbital were mixed with 13.8 mls of 0.1 N HCl for a pH of 8.6
- IEF agarose (Pharmacia).
- antigen preparation: prepared from a SV5 or PF2 infected VERO cell culture.
- 'first antibody': Rabbit anti-human (or mouse) antibody.
- $^{125}$I linked to Protein A (Amersham)
- washing solution: 0.5 % (V/v) NP 40 in PBS.
- 10 ml pipette
- glass plate and casting frame.
- gel drier.
- rocker.

b/ Method: As in the immunoblotting method, 2 types of gels were prepared. If oligoclonal bands had to be visualised on the gel, the gel was purely made of agarose in barbital buffer. If specific-virus oligoclonal bands had to be visualised, the virus was incorporated into the gel.

1. Preparation of agarose gel (with or without antigen):
- SV5 or PF 2 cell pack antigens were prepared from infected VERO cell cultures (see section MM8).
- The infected cells were suspended in PBS to 10 % (v/v) and sonicated for 30 seconds on ice.
- The infected cell suspension was clarified by centrifugation (1000 g for 5 minutes).
- Control antigens were prepared from non infected VERO cells in the same way.
- The gel was made by mixing 1.5 % (w/v) agarose in barbital buffer and the mixture was allowed to set.
- Antigen (virus)-containing gel was prepared by heating the agarose gel (1.5 % (w/v) agarose in barbital buffer) to
56 °C. 100 µl of the antigen preparation was added per 10 mls of gel.
- The mixture (gel with antigen or alone) was layered (0.8 mm) with a 10 ml pipette onto an alcohol cleaned glass plate (8x8 cm) already covered ('primed') with a layer of gel and allowed to set. Once the gel was set, its edges were trimmed.

2. Isoelectrofocusing & imprint immuno-fixation
- Immediately after IEF of the CSF on polyacrylamide gel (see section MM1), the gel plates containing virus were placed (gel facing downwards) in a horizontal position in direct contact with the electrophoresis gel. (a small volume of de-aerated buffer was layered between the gels to prevent trapping of air bubbles.
- Immunodiffusion (gel to gel overlay) was carried out in a moist chamber for 5 minutes at room temperature.
- The 2 gels were separated by a gentle sliding movement after a brief submersion in barbital buffer.
- The antigen containing gel plate was repeatedly washed in PBS and pressed under filter paper.
- 'First antibody' was incubated with the gel (1 in 500 dilution in 0.2 % BSA-PBS) for one hour at room temperature on a 'rocker'.
- The gel plate was washed in PBS 3 times.
- It was then incubated with $^{125}$I linked to Protein A for 1 hour on a rocker.
- The gel plate was washed with 0.5 % NP 40 in PBS, followed by a final wash in distilled water. The gel plate was pressed under filter paper and the gel was dried.
- The gel was then exposed to a X ray film at - 70 °C overnight.
- the X ray film was then developed.
**MM6: PREPARATION OF VIRUS INFECTED CELLS AND IMMUNOFLUORESCENCE SLIDES.**

The method will be described for VERO (Monkey kidney cells) cells and SV5.

**a/ Material** - stock of VERO cells.
- 75 cm³ 'Cel-cult' culture flask.
- G-MEM medium (Gibco).
- newborn calf serum (NBCS).
- PBS
- fixative buffer: 5 % (v/v) formaldehyde
  2 % (w/v) sucrose
  in PBS.
- permeabilisation buffer: 0.5 % (v/v) NP 40
  10 % (w/v) sucrose
  in 1 % NBCS- PBS.
- 5% CO₂ incubator.
- trypsin-EDTA solution:
  0.05% trypsin, 0.02 % EDTA, in PBS, pH 7.3,
  filter-sterilised and kept at -20 °C.
- 12-well immunofluorescence slides (Hendley-Essex Ltd)
- coverslips (Arnold R. Ltd, London)
- square petri-dishes (Sterilin).
- sterile Pasteur pipette.
- Class II hood.
- haemocytometer slide.

**b/ Method: 1. Trypsinisation of the VERO cells**
- Stock of VERO cells: cultured in G-MEM medium
  supplemented with 10 % NBCS, in 75 cm³ 'Cel-cult' culture plastic petri dishes.
- Under sterile conditions, the cells were washed twice with
  5 ml of trypsin-EDTA solution. Approximately, 2 mls of
  trypsin-EDTA solution was left on the cells. The flask was
  then incubated at 37 °C for 2-3 minutes. When most of the
  cells were seen to have been trypsinised optimally (the cells
  were of circular shape, attached loosely to the surface of
  the plastic flask), the flask was agitated gently and warm 10
  % NBCS medium was added to the flask.
- The cells were counted and resuspended to a concentration of 2.10^5 per ml.

2. Preparation of the infected immunofluorescence slides.
- Autoclaved 12-well Micropore immunofluorescence slides were transferred under sterile conditions in square petri dishes (3 immunofluorescence slides per square petri dish). The top left corner of each slide was labelled.
- 70 μl of the cells suspension containing 2.10^5 cells/ml was transferred to each well of the immunofluorescence slide.
- The slides were incubated overnight at 37 °C, in 5% CO2 atmosphere.
- When the cells had reached confluence (after 24 hours) the medium was removed with a Pasteur pipette connected to a vacuum line.
- 70 μl of 1/20 dilution of the virus seed in 2 % NBCS G-MEM was added in each well. NB: virus manipulation was done in predetermined controlled areas.
- The slides were incubated for 1-2 hours at 37 °C, in 5% CO2 atmosphere. 30 mls of 2 % NBCS G-MEM were then added to each square petri dish.
- The slides were incubated for 24 hours at 37 °C, in 5% CO2 atmosphere. This incubation time was optimal for immunofluorescence: plaques of viral infection cohabited with area of cells not infected with the virus thus providing an internal negative control. Uninfected slides were also prepared as negative control.

3. Fixation, penetration and storage of immunofluorescence slides.
- Slides were washed in 1 % NBCS-PBS twice,
- Incubated for 10 minutes in fixative buffer, and washed once in 1 % NBCS-PBS.
- They were then incubated with the permeabilisation buffer for 5 minutes, and washed one to three times with 1% NBCS-PBS.
- The slides were stored in 1 % NBCS-PBS + azide (0.01%) at + 4 °C for weeks.
MM7: INDIRECT IMMUNOFLUORESCENCE TECHNIQUE.

a/ Material - Positive and negative antigen slides; the method will be described for VERO cells infected with SV5.
- 'First antibody': a monoclonal anti-SV5 antibody made in mice or a polyclonal antibody made in rabbit or a human serum.
- 'Second antibody' depended on the species of the first antibody. The second antibody used in the experiments was 'conjugated' to fluorescein.
- VERO cell stock grown in 75 cm³ culture flask.
- 'Rocker' at 37 °C.
- syringe with filter holder and filter (e.g Millipore, cat GSWPO1300, type GS, pore 0.22µ)
- Bench centrifuge with 1.5 ml Eppendorf's tubes.
- 1 % NBCS-PBS.
- forceps and tissues.
- Citifluor® anti fading agent (University College, London).

b/Method: 1. Preabsorption of the 'conjugate' (second antibody): the 'second antibody' was absorbed with the cell line that was used for the preparation of the infected slides. This reduced the non specific background which could have occurred with some conjugated antibodies. When the 'second antibody' was 'absorbed' the optimum dilution had to be determined with positive controls.
- VERO cells in a 75 cm³ culture flask were fixed and permeabilised as described in section MM6 of "Preparation of virus infected cells immunofluorescence slides" and then washed 3 times with 1 % NBCS-PBS.
- Residual PBS was removed with a Pasteur pipette.
- 3 mls of the 'second antibody' solution diluted 1/100 in PBS was incubated with the VERO cells in the flask.
- The flask was rocked gently at 37 °C for 1 hour.
- The 'conjugate' solution was aspirated from the flask and transferred into Eppendorf tubes which were then centrifuged at 12,000g for 2 minutes (in order to remove the cell debris from the conjugate solution).
- The conjugate solution was filtered through a 0.22 μm pore filter and kept at +4 °C for some weeks.

2. Indirect immunofluorescence procedure:
- Immunofluorescence slides (infected and uninfected) were washed once with 1 % NBCS-PBS.
- The slide without cells as well as the edges of the slide and the spaces between every wells were dried carefully with the corner of a tightly folded tissue.
- 10 μl of required dilutions of the first antibodies were placed in their respective wells in accordance with a predetermined pattern.
- The slides were placed in moist square petri dishes and incubated for 30 minutes to 1 hour at room temperature.
- The slides were then washed once with PBS quickly but gently and then 3 times with 1 % NBCS-PBS (5 minutes each time).
- The slides were then dried as previously described with a tissue paper.
- 10 μl of the appropriate dilution (and species) of second antibody (ies) were then incubated in their respective wells. At least one well was needed with no 'first' or 'second antibody' and was therefore incubated with 1 % NBCS-PBS. The incubation time was 30 minutes to one hour at room temperature, in the dark.
- The slides were then washed 3 times with 1 % NBCS-PBS.
- the slides were dried as described before with a tissue and 10 μl of Citifluor was applied between the wells, in the middle of the 2 rows. The coverslips were gently applied to the slides.
- The slides were visualised using the fluorescence microscope.

- The first antibody incubation step is replaced by an incubation with 10 μg/ml of DAPI in PBS and the second antibody incubation step is omitted.
a/ Material - VERO cells
- virus seed: $10^6$ pfu/ml (pfu (plaque forming unit))
- roller bottles and 'Hot' room.
- G-MEM medium (Gibco)
- Ultracentrifuge (Beckman)
- $30\%$ sucrose in PBS.
- new born calf serum (NBCS)

b/ Method - VERO cells were grown in 75 cm$^3$ cell culture flask and then expanded in 'roller' bottles in G-MEM with $10\%$ NBCS.
- At confluence, the cells were washed with G-MEM without calf serum and then infected with $1/20$ virus seed in G-MEM without calf serum.
- Cells were grown, without calf serum, for 30-35 hours at $37^\circ C$.
- The supernatant was then collected and centrifuged at low speed in order to clear cell debris (5000 g).
- The supernatant was centrifuged over a $30\%$ sucrose gradient in PBS at $24\,000$ rpm ($110,000$ g) with a SW 28 rotor on a Beckman centrifuge.
- The pellet was collected and suspended in PBS as the purified virus preparation.
MM9: SDS PAGE ELECTROPHORESIS.

a/ Material: - 'antigen' sample
- sonicator (MSE probe)
- SDS PAGE marker (BCL)
- minigel rack (LKB)
- power pack (LKB, Multiphor II electrophoresis unit)
- minigel plates, spacers (0.75 mm), combs (0.75 mm) and
  bull-dog clips (all from LKB).
- Hamilton pipette.
- electrode buffer:
  30 g TRIS.
  144 g Glycine.
  50 mls of 20 % (w/v) SDS.
  deionised H₂O to 10 liters.
- chemicals for separating and stacker gels:
  10 % (w/v) SDS
  Bis-acrylamide (50:0.235) solution for separator gel:
    50 g of acrylamide + 0.235 g of MM-
    methylbisacrylamide
    dissolved in up to 100 mls of distilled water
  Bis-acrylamide (50:1.33) solution for stacker gel:
    50 g of acrylamide + 1.33 g of MM-
    methylbisacrylamide
    dissolved in up to 100 mls of distilled water.
  1 M Tris buffer pH 8.6
  1 M Tris buffer pH 7
  TEMED
  ammonium persulphate (10 mg/ml of deionised H₂O)
  freshly made the same day.
- separating gel (for a 15.2 % (w/v) acrylamide gel)
  standard large size gel (for a minigel the quantity are
  divided by 3)
  0.3 ml of 10 % (w/v) SDS.
  9.5 ml of Bis-acrylamide (50:0.235)
  11.2 ml of 1 M tris buffer pH 8.6.
  6 ml of deionized H₂O.
  30 μl of TEMED.
  3 ml of ammonium persulphate
- stacker gel (for a minigel the quantity can be divided by 5).

0.1 ml of 10 % (w/v) SDS
1 ml of Bis-acrylamide (50:1.33)
1.25 ml of 1 M Tris buffer pH 7.
6.65 ml of distilled water.
10 μl of TEMED.
1 ml of ammonium persulphate.

- disruption mixture (x4 strengh):
  1 ml of 20 % (w/v) SDS.
  1 ml of 1 M Tris buffer pH 7
  1 ml of 8- mercaptoethanol
  1 ml of 50% (v/v) glycerol in H2O
  + bromophenol blue (until desired colour obtained)

- stain solution:
  20 % (v/v) acetic acid
  20 % (v/v) methanol

- destain solution:
  10 % (v/v) acetic acid
  20 % (v/v) methanol

- 3 MM Whatman paper, cling film
- gel dryer.

b/ Method: 1. Preparation of the SDS PAGE gel:
- The glass plates, comb and spacers were thoroughly cleaned with detergent and distilled water.
- The glass plates were cleaned with 70 % (v/v) alcohol, polished with tissue and then the gel plates and spacers were assembled together. They were then taped with yellow waterproof electrophoresis tape in order to avoid leakage. The assembled gel plates were fixed in a vertical position with bull-dog clips.
- The separator gel solutions were mixed together in a glass vacuum flask. the flask was air-vacuumed and the contents pipetted between the 2 plates with a Pasteur pipette until the correct level was reached.
- Distilled water or isobutanol was added to the top of the gel in order to provide a straight line at the top of the separator gel. The gel was allowed to set (a sample of the
mixed separator gel solution was kept in a container to checked when the gel was set).
- The distilled water or the isobutanol was poured off completely and the stacking gel solutions were mixed together and pipetted over the separator gel.
- the comb was inserted into the liquid stacking gel between the two plates and the stacking gel was allowed to set (a sample was kept in a small bottle to confirm that the stacker had set).

2. Electrophoresis:
- The bottom tank was filled with electrode buffer.
- The gel apparatus was connected to the power pack
- For a minigel, the gel was run at 40 V until the line reached the separator gel and then at 120 V to the bottom of the separator gel.
- At the end of the electrophoresis, the plates were separated and the bottom left-hand corner of the gel was cut out to mark the side of the first track. The gel was carefully transferred either
  1. to the semi dry blotter apparatus (see section western blotting MM10) or
  2. placed in stain for 1 to 2 hours on a 'rocker'.
- The gel was then destained with multiple changes of the destain solution, placed on a blotting paper and covered with cling film, and dried in a gel dryer under vacuum.
MM10: WESTERN-BLOTTING PROTOCOL.

a/ Material:- SDS PAGE gel (see section MM9)
- semi dry blotter (Novablot 2117-250 LKB, electrophoresis transfer kit)
- power pack (LKB Multiphor II, electrophoresis unit)
- forceps, scalpel, ruler and gloves.
- Whatman blotting paper M3.
- nitrocellulose paper: Schueller and Schleicher, 0.2μm pore size.
- anode buffer 1: 0.3 M Tris (for 500 ml, 18.171 gr of Tris)
  20 % (v/v) methanol
  pH 10.4
- anode buffer 2: 0.025 M Tris (for 500 mls, 1.515 gr)
  20 % (v/v) methanol
  pH 10.4
- cathode buffer:0.025 M tris
  20 % (v/v) methanol
  40 mM 6-amino-n-hexanoic acid (for 500 ml, 2.642 gr) pH 9.4
- Marvel®
- 'first antibody'
- 'second antibody'
- NP 40
- Naphthalene black stain solution.
  0.01 % (w/v) naphthalene black powder
  20 % (v/v) acetic acid
  20 % (v/v) methanol in water.
- Destain solution.
  20 % (v/v) acetic acid
  20 % (v/v) methanol in water
- 3’,3’-Diaminobenzidine (DAB tetrahydrochloride):
- 50 mM Tris (pH 7.6).
- 30 % (v/v) H₂O₂ (H₂O₂ supplied as a 30 % solution (BDH) and kept at +4 °C)
b/ Method: 1. Electroblotting transfer of proteins from SDS PAGE gel to NTP.
   - The SDS PAGE was run (see SDS PAGE section MM9) with the different antigen preparations to be tested, or, with one antigen with a one-tooth-comb.
   - At the end of the electrophoresis, the semi dry blotter was cleaned thoroughly with distilled water.
   - 6 Whatman blotting papers were soaked with anode 1 buffer and applied to the anodic plate of the blotter (excess of buffer was avoided). The Whatman paper was 0.5 cm larger on each side than the NTP sheet.
   - In the same way, 3 Whatman papers were soaked with anode 2 buffer.
   - The nitrocellulose paper was cut out (the NTP was handled carefully with scalpel and forceps) of the appropriate size (the NTP was 0.5 cm larger on each side than the SDS PAGE).
   - The NTP sheet was placed on the top of the Whatman papers and was humidified by absorption of the anode 2 buffer from the top Whatman paper.
   - The SDS PAGE plates were separated carefully and the gel normally adhered to one plate. The gel was covered with a dry Whatman paper. The Whatman paper was then humidified by absorption over the cathode buffer (the gel was not in contact with the cathode buffer).
   - The gel, with the Whatman paper on top, was placed over the NTP - the bottom left of the SDS-PAGE gel in contact with the bottom left of the NTP sheet.
   - A 10 ml pipette was rolled over the top Whatman paper (to minimise bubbles) and 8 more Whatman papers, soaked in cathode buffer, were placed over the top Whatman paper.
   - The electrotransfer was carried out at 0.8 mA/cm² (of the Whatman paper) for 1 hour at room temperature.
   - At the end of the electroblotting, the SDS PAGE was stained with coomassie blue to verify that proteins had been transferred. If the antigen transfer preparation was a single antigen preparation (loaded with a one tooth comb) the sides of the NTP paper were also stained in naphthalene black solution (1 minute in naphthalene stain, 1 minute in
SDS-PAGE destain and 1 minute in water before drying between 2 blotting papers) in order to locate exactly the location of the transferred proteins (for an example see figure R8-1 in Results section: the western blotting study).

2. 'Western' blotting visualisation.
- The NTP sheet was 'blocked' for 2 hours in 10 % (w/v) Marvel in PBS on a 'rocker' at room temperature.
- The NTP was washed briefly in PBS.
- It was then incubated with the appropriate dilution of the 'first antibody' in 5 % (w/v) Marvel in PBS (e.g 1/1000 for a test serum). The incubation was for 2 hours or overnight at room temperature on a 'rocker'.
- The NTP was then washed
  10 minutes in PBS
  2 x 10 minutes in 0.05 % (v/v) NP 40 in PBS
  10 minutes in PBS.
- The next stages of the protocol were done in a dark container.
- The 'second antibody' (peroxidase conjugate) was incubated with the NTP for one hour at room temperature.
- The NTP was then washed
  10 minutes in PBS
  2 x 10 minutes in 0.05 % (v/v) NP 40 in PBS
  10 minutes in PBS.
- The visualisation of the reaction was done with DAB: 6 mg of DAB was dissolved in 10 mls of 50 mM Tris (pH 7.6) and filtered through Whatman 1 paper. Just before development, 10 µl of 30 % (v/v) H2O2 was added. The blot was developed on a 'rocker', at room temperature, in a dark container. The reaction was stopped with PBS [Harlow E. et al, 1988].
MM10': DOT BLOT FOR PROTEIN CONCENTRATION.

a/ material - Bovine serum albumin (BSA):
   1mg/ml in PBS + 0.01 % azide, kept at +40°C.
   - Nitrocellulose paper: Schueller and Schleicher, 0.2μm pore size.
   - Naphthalene black stain solution.
     0.01 % (w/v) Naphthalene black powder
     20 % (v/v) acetic acid
     20 % (v/v) methanol in water.
   - Destain solution.
     20 % (v/v) acetic acid
     20 % (v/v) methanol in water
   - 96 Microtiter plate.

b/ Method
A microtiter plate is labelled in order to allocate one row of this microtiter plate to each protein solution to be tested. The first row is allocated to the standard solution (BSA). Ten μl of PBS are pipetted into each necessary well. Ten μl of the standard solution of BSA (1mg/ml) are pipetted into the first well of the microtiter plate and double dilutions of this standard solution are made along the first row of the microtiter plate. Then the solutions to be tested are processed similarly, each solutions requiring a row. Two μl from each well are then spotted on a nitrocellulose paper so as to compare the different protein solutions (each solutions requiring a line commencing with the more diluted dilution of the solution). The different dilutions of the protein solutions are allowed to absorbed unto the nitrocellulose paper for 1 minute. The nitrocellulose paper is then stained for one minute in the stain solution destained for 1 minute in the destain solution and washed for 1 minute in water. The nitrocellulose paper is then dried between 2 blotting papers and the concentrations of each protein solutions calculated by comparing the known concentration of the standard solution (BSA= 1mg/ml, 2 μg of BSA correspond to the first spot)
MM11: T CELL PROLIFERATION ASSAY.

a/ material- sterile plastic universal.
- heparin preservative-free (Leo Ltd, 1 million unit/ml).
- venepuncture material (butterflies, micropore tape, scissors, swabs, tourniquet and labelled universal containers with heparin).
- sterile Pasteur pipette.
- sterile 10 ml centrifuge tubes (white cap, V bottom).
- a set of multidispensing pipettes (Gilson pipettes)
- sterile tips for Gilson pipettes
- electric multidosage pipette.
- test tube holders (one for universal and one for 10 mls tubes)
- Class II hood with 10 mls sterile pipette and automatic dispensing pipette system.
- Ficoll- Hypaque® (Pharmacia), sterile.
- Hank's medium, pH 7.4, sterile, for diluting the blood and washing the mononuclear cells.
- swing-out centrifuge for 10 ml and universal tubes
- glutamine solution (x 100): 29.2 mg/ml of double distilled water filtered and sterilised.
The solution was aliquoted and stored at - 20 ºC
The final concentration (x 1) was 2 mM
- Penicillin / streptomycin solution (x 100):
  The final concentration (x1) was 5 UI/ ml of penicillin and 75 µg/ml of streptomycin.
e.g in the laboratory the Penicillin/streptomycin solution was prepared as follows:
In 400 ml of sterile deionized distilled water
Crystapen (1 million UI/ vial) 2 vials
Streptomycin (1g/vial): 3 vials
The solution was aliquoted and stored at - 20 ºC
- hepes solution (x 100): 292 mg/10 ml of deionized distilled water
  buffered to pH 7.4 ,
  filtered, sterilised.
The solution was kept at + 4 ºC
  The final concentration (x 1) was 10 mM
- β-mercaptoethanol solution (β-MCE): (x 100 solution)
β-MCE (Sigma) special cell culture grade, was kept at + 4 °C aliquoted e.g 100 µl aliquots. An aliquot was used only once.  5 µl of β-MCE were added to 15 ml of PBS. The solution was filtered, sterilised and kept at + 4 °C for 3 weeks.

- T cell medium: filtered sterilised.
  for 20 mls
  RPMI 1640 (Gibco) 19.2 mls
  glutamine solution (x100) 200 µl
  Penicillin/Streptomycin (x100) 200 µl
  Hepes (x100) 200 µl
  β-MCE solution 200 µl

- Trypan blue solution:
  Solution A: 0.2 % (w/v) + azide
  Solution B: 4.25% (w/v) + azide.
  1/2 working concentration: 2 volumes of solution A + 0.5 volume of solution B. Add 10 µl of the cells solution for counting to the same 10 µl volume of trypan blue solution.

- sterile 96 wells plates (U bottom) with sterile lids.
- sterile Eppendorf tubes for dilution of antigens and mitogens.
- Eppendorf racks
- antigen preparation : diluted in T cell medium.
- mitogen preparation : diluted in T cell medium.
- $^3$H thymidine(methyl-$^3$H), (Amersham) , 1 m Curie/ml.
- cell harvester with vacuum pump on line (Ilacon, Tunbridge)
- glass fiber paper (Ilacon).
- scintillation fluid : home-made for 4 liters
  2666 ml xylene (kept in cold room)
  1333 ml triton x100
  0.48 g POPOP (1.4 Di-2-5 phenyloxazoly-Benzene)
  24 g PPO (2.5 -Diphenyloxazole)
- scintillation vials and pico-vial
- forceps and scalpels for radioactive work.
- β counter A300 Canberra Packard liquid scintillation fluid.
b/ **Method:** (see figure MM11-1 for outline of the technique)

- Peripheral blood was collected by venepuncture in a sterile plastic universal with preservative-free heparin (20 units/ml of blood) and used within 4 hours.
- The blood was mixed with the heparin and left for at least 30 minutes at room temperature and then diluted 1 in 2 with warm (18-22 °C) Hank's medium. The method described was for 10 mls of blood, diluted 1 in 2 to obtain a Hank's-blood mixture of 20 mls and overlaid on the
  : in each labelled 10 ml centrifuge tube, 3 ml of ficoll-hypaque was overlaid with 7 mls of blood. Thus 3x10 mls centrifuge tubes were required.
- The tubes were centrifuged at 800 g for 20 minutes (18-22 °C) (the centrifuge rotor was slowly accelerated and decelerated).
- Mononuclear cells were recovered at the interface with a sterile Pasteur pipette. The upper plasma layer was not removed before recovering the mononuclear band. The mononuclear cells were collected from the periphery and cross sectional area of the centrifuge tube. From each of the 3 tubes, they were transferred into 3 respective new 10 ml tubes.
- Mononuclear cells were washed with warm Hank's solution at 800 g for 10 minutes. After centrifugation, the supernatant was discarded and the pellet of cells was resuspended gently and the contents of the 3 tubes transferred to 2 tubes (one tube was discarded).
- The cells were washed a second time with warm Hank's solution, at 400 g for 10 minutes and again the pellet was resuspended gently and pooled in one tube.
- After another washing with Hank's solution at 400 g for
Peripheral blood → Ficoll gradient 800g 20' → Washing of the mononuclear cells x3 400g 10' → Resuspended at 2x10^6 cells/ml → Harvest on glass fiber paper → Pulse with ^3H^3Thymidine the last 18 hours → Incubation of 100μl of cell suspension/well with antigens, medium or mitogen → ^3H^3Thymidine Culture 3 - 7 days 37°C 5% CO₂ → Count in scintillation fluid

Figure MM11-1: Outline of the T cell proliferation assay
10 minutes, the cells were resuspended in one ml of T cell medium containing 10% of sterile serum (for human studies, the serum was AB+ from human source or FCS).
- The cells were then counted with the Trypan blue solution and only live mononuclear cells were taken into account (normally more than 90% of the mononuclear cells were viable).
- The cells were resuspended to a final concentration of 2.10^5 per 100 μl (2.10^6 per ml) in 10% AB + T cell medium.
- 100 μl of the cell suspension was added to predetermined wells of a 96 well plate.
- 100 μl of antigen/mitogen or medium dilutions were added to each well, in triplicate for each test sample, in accordance with a preorganised pattern (for an example see figure MM11-2)
- The 96 well plate was then incubated at 37 °C in a 5% CO2 atmosphere for 3-7 days.
- 0.5 μCi ³H thymidine was pulsed into each test well 18 hours before the harvesting: the ³H thymidine was diluted in T cell medium in 20 μl of T cell medium per well.
- After 18 hours, the cells were harvested onto glass fiber paper.
- Each glass fiber paper was placed into a plastic pico vial which was itself placed into a glass vial scintillation fluid. 5 ml of scintillation fluid was added to each pico vial.
- The vials were then counted in a β counter and the results analysed.
Figure MM11-2: T cell proliferation assay pattern of the 96 well plate used for the 'NTP and T cell proliferation assay. All test samples were done in triplicate.
MM12: NITROCELLULOSE T CELL TECHNIQUE
PROTOCOL.

a/ Material (in chronological order as used)

- Antigen (Ag) preparation: the concentration of proteins was necessary for the calculation of the protein load on the gel. The loading concentration was determined as a function of the protein concentration of the Ag preparation and of the number of proteins in the antigen preparation (e.g. mycobacteria preparation was a very heterogeneous antigen preparation in comparison with purified tetanus toxin).
- disruption buffer for SDS PAGE (see section SDS PAGE, MM9).
- solutions for the gel casting (see section SDS PAGE).
- spacers and comb (0.75 mm wide) cut for this experiment.
- gel apparatus (minigel LKB®). This technique was carried out using a minigel and therefore all the data and calculations will be standardised for this type of gel.
- power pack for SDS PAGE and for the electroblotting (see corresponding sections).
- nitrocellulose paper (NTP): (Shueller and Sleich, porosity 0.2 μm).
- semi-dry electroblotter apparatus (Novablot® 2117-250 LKB electrophoretic transfer kit)-with material for blotting: Filter papers, anodes (1 and 2) and cathode buffer and NTP (see Western Blotting section MM10).
- ruler and pencil.
- sterile scalpels (at least 2 or 3)
- sterile forceps (at least 2 pairs)
- naphthalene black solution, with destain, water and filter paper to dry stained NTP.(see Western Blotting section MM10)
- polystyrene board with needles to dry the nitrocellulose paper for blotting experiments.
- sterile square dishes for tissue culture
- vacuum line with clean Pasteur pipette.
- fridge or cold room (if possible near the cell culture lab) where a minifuge for Eppendorfs tubes (MSE microcentrifuge at 10,000 g) was placed and where NTP sheets pined over the polystyrene board were dried.
- Class II hood with at least two power points available.
- Glass vials with lids(Clear neutral glass vials Adelphi® Tubes, Haywards Heath, with 15.5 mm white screw caps).
- Magnetic fleas: one magnetic flea was placed inside each glass vial (magnetic followers for oxygen electrode Rank Bros Ltd, Cambridge). The glass vials with the magnetic fleas were autoclaved.
- sterile Pasteur pipettes.
- DMSO : 100ml bottle (BDH), kept at + 4° C, lid tightly closed.
- a set of multidispensing pipettes e.g Gilson pipettes.
- test tube vortexer (whirlmixer).
- peristaltic pump (Minipuls 2, Anachem)
- silicone plastic tube to fit the peristaltic pump:
  The 2 ends were wrapped in foil and the entire tube then autoclaved in a plastic beaker covered with foil.
- a stand, small enough to fit inside the hood, to hold the silicone tube.
- magnetic stirrer with plug
- filter-sterilised 0.05 M carbonate buffer Ph 9.6 in plastic universal. The recipe of the carbonate buffer was as follows [Hudson L. et al, 1989]:
  Stock A: 0.2 M anhydrous sodium carbonate, Na2CO3
  2.12 g / 100 mls
  Stock B: 0.2 M sodium hydrogen carbonate
  1.68 g / 100 mls
  For pH 9.6, 13 mls of solution A and 37 mls of solution B were added to 150 mls of distilled water and then the pH was checked.
  After dilution of 1/4 a solution of 0.05 M was obtained.
- autoclave tape.
- Eppendorfs 2 sorts: 1) 400 µl for aliquots (the number needed was calculated in function of the number of bands ; in general, 10 aliquots/bands were sufficient)  
  2) 1.5 mls for washing
Both types of Eppendorfs were autoclaved.
- Eppendorf racks (2 or 3 depending on the number of aliquots)
- RPMI 1640 for washing.
- minifuge(s) (12 Eppendorfs were centrifuged at the same time)
- test tube support with teeth (Endicott Seymour, Ann Arbor, Michigan, U.S.A.)
- T cell medium with penicillin/streptomycin, hepes pH 7.4, glutamine and β-mercaptoethanol (if used in the T cell medium), cell culture grade e.g from Sigma (see section MM-11).

2. METHOD

2 techniques were used - the spotting technique and the western blotting techniques. These 2 techniques differed only in the way the antigen(s) was applied to the NTP but both techniques were the same after this stage. In the protocol below, the sign * marks the stage at which the 2 techniques begin to be identical and remain so until the end of the procedure.

A/ SPOTTING TECHNIQUE:
- Antigen preparation: protein content was calculated by the dot blot technique (with albumin solution of 1mg/ml as standard).
- A piece of NTP sheet (4 x 4 cm) was prepared.
- Rectangles of 20 mm² (5 x 4 mm) were drawn using a fine pencil and a ruler on the NTP. The number of these rectangles depended on the number of antigen-NTP particles required.

See figure MM12-1
Rectangles were labelled for the corresponding antigen. At least one square was needed for the negative control: e.g PBS spotted on NTP / buffer of the antigen preparation without antigen spotted on NTP.
Figure MM12-1: Spotting 'nitrocellulose technique'. Rectangles corresponding to the different antigens to be prepared are drawn on nitrocellulose paper.

- The NTP was then soaked (only one side) with PBS to humidify. The NTP was taken out of the PBS when its colour had changed (thus the NTP was not be completely covered with PBS).
- The NTP was then dried in order to dispose of the excess PBS.
- 10 μg of antigen were spotted on each rectangle. If the concentration of the Ag preparation was not very high, great care was taken to avoid spillage over the boundaries of the rectangle; for example a 20 mm² rectangle received easily 5 to 10 μl corresponding to an Ag preparation of a concentration of 2 to 1 mg/ml of proteins respectively. Below 1mg/ml, the volume of Ag preparation required to achieve 10 μg per rectangle was too great to avoid spillage. In this case the Ag was spotted over a period of time to allow absorption of the Ag solution by the NTP.
- The NTP was dried at + 4° C using a polystyrene board with needles:
  see figure MM12-2.

**Figure MM12-2:** The nitrocellulose paper where antigen have been spotted is dried on a polystyrene board.

- When the NTP was completely dry, the NTP was placed on a sterile square petri dish in a hood flow cabinet.
- Each rectangle was then processed separately, starting with the negative control. Each rectangle was cut out and then cut into small pieces of 1 to 2 mm square using sterile forceps and scalpels.

***AT THIS STAGE THE 2 TECHNIQUES (SPOTTING AND BLOTTING BECOME IDENTICAL)***

- The pieces were transferred with the tip of a sterile Pasteur pipette into a labelled (DMSO can erase marker
ink) sterile glass vial, which contained a sterile magnetic flea.

see figure MM12-3.

Figure MM12-3: The pieces of nitrocellulose are transferred into sterile glass vials.

- All rectangles were processed in the same way and transferred into their corresponding labelled glass vials.
- 500 µl of DMSO were added (the quantity of DMSO depended on the quantity of NTP in the vial- see the section results)
- the vials were closed with sterile lids.
- the NTP pieces dissolved in the DMSO and a colourless liquid was left.
When the pieces had disappeared, the DMSO was stirred for 30 seconds to 1 minute with a magnetic stirrer. A constant slow speed was maintained.
- the glass vials were left at room temperature for one hour and were stirred every 15-20 minutes.
- after this incubation period, sterile condition were strictly adhered to (the DMSO kills most, if not all germs and therefore after this stage the preparation was considered sterile).
- under the flow cabinet, the peristaltic pump was fitted with the autoclaved siliconised plastic tube hanging from the stand (care was taken to keep the 2 ends of the tube sterile as they hung)
- One end of the tube was submerged in the filtered sterilised carbonate buffer contained in a sterile plastic universal beaker.
The plastic universal was taped inside a small beaker to avoid accidental spillage.
- The glass vial was taped at the middle of the magnetic stirrer.
- The magnetic stirrer was set at a fast speed; this speed was kept constant for each vial and between vials in order to standardise the method.
- The carbonate buffer was then added to the vial at a speed of 0.8 ml/minute through the silicone tube using the peristaltic pump.

see figure MM12-4
- 750 μl were added if the original volume of DMSO was 500. For an original volume of 250 μl, 500 μl of carbonate buffer were sufficient.
Figure MM12-4: System for dispensing the carbonate buffer into the vials containing the pieces of nitrocellulose.
- Small particles of NTP precipitated and the liquid in the vial became cloudy.
- The liquid containing the NTP particles was transferred with a sterile Pasteur into a sterile labelled 1.5 ml Eppendorf.
- The Eppendorf was centrifuged at 10,000 g for 10 minutes
- The NTP particles formed a pellet at the bottom of the Eppendorf tube. The colourless supernatant was sucked up carefully using a sterile Pasteur pipette connected to the vacuum line.
- 50 µl of RPMI was then added to the Eppendorf tube.
- The closed Eppendorf tube was then "run to, missing the fro" 5-10 times over the teeth of the test tube support. This helped to resuspend the particles in the RPMI.
- 1 ml of RPMI was then added and centrifugation, washing, resuspension were repeated 3 times but, unlike the first centrifugation, the next centrifugations were done at + 4° C.
- After the last washing, the particles were resuspended in complete T cell medium instead of RPMI.
- Normally 20 mm² NTP was resuspended in 1 ml of complete T cell medium and aliquoted in 50 ml aliquots.
- The aliquots were stored at - 70° C and could be kept for months.
B/ THE BLOTTING TECHNIQUE.

The Ag in this technique was run on a SDS PAGE gel and then blotted onto NTP by electrophoretic blotting with a semi dry blotter. In this protocol, the details relevant to the NTP T cell technique only will be described.
- A comb was cut out as a function of the template of the experiment. Usually, 3 tracks for Ag were needed as well as the tracks for the marker.
  1. one track for the negative control: disruption mixture alone
  2. one track for the Ag to be tested
  3. one track for an Ag known to produce a positive response in an available control (in our case tetanus toxin or adenovirus).

The tracks corresponding to the Ag measured 12 mm in width.
The tracks corresponding to the marker measured 3 mm only.

See figure MM12-5 for a template of a comb.

![Template of the comb used for loading the antigens on the SDS-PAGE gel](image)

**Figure MM12-5**: Template of the comb used for loading the antigens on the SDS-PAGE gel.

- The loading concentration varied with the antigen preparation and the number of proteins bands recognised on a gel. Normally the loaded amount of protein for a
single band was between 1.5 - 3 µg, for a 12 mm minigel track (see figure MM12-6).
- The gel was run (see SDS PAGE section MM9) and transferred onto NTP by electroblotting (see electroblotting section MM10)
- During the preparation of the electroblotting, specific precautions and preparations were followed:
  1. When the 2 gel plates were separated after electrophoresis, the SDS page gel was normally stuck to one of the plates. A dry sheet of Whatman paper was then applied to the gel.
  2. The tracks of the SDS PAGE gel were located on both sides - top end and bottom end - of the gel. This was straightforward with the top end as the indentation of the comb was still visible in the stacking part of the SDS PAGE gel. At the bottom end, the boundaries of the tracks were guessed by projection from the top end of the tracks. A transparent ruler was used on the assumption that the tracks ran perpendicular to the gel.
  3. When the limits of the tracks were established, holes in the Whatman sheet were made, with a small needle, corresponding to the 4 corners of the track.
  4. The Whatman paper was then soaked in cathodic electroblotting buffer (the SDS PAGE must not be in contact with this buffer) and then the Whatman paper with the SDS PAGE gel was applied to the NTP.
  5. The holes, already made in the Whatman paper, were used as a guide to mark the peripheries of each track on the NTP sheet. Again a small needle was used for this.
  6. The end of the electroblotting procedure was similar to standard electroblotting method.
- After transfer, the NTP was carefully cut to give vertical lanes measuring 6 mm of width spanning the track of the sample. The NTP on both sides - which were labelled - was then stained (3 mm each side of the lane) in order to locate the proteins of interest.

See figure MM12-6

- 2 mm bands were then cut out from the 6 mm width lane. Each bands corresponded to a region of the track to be tested as a 'specific antigen'.

120
Each band was then processed in a similar fashion to the rectangles of the spotting technique. The quantity of DMSO necessary to produce an optimum precipitation at the end of the procedure, was 250 μl for each 12 mm² band. However, a mock experiment was run to determine this quantity (see the spotting technique result section).

Figure MM12-6: Blot of Tetanus Toxin (TT) used for the preparation of nitrocellulose particles (NCP) coated with TT. TT was transferred on nitrocellulose (Ntcp) from an SDS minigel. A comb measuring 12 mm in width was used to load the proteins on the gel. The Ntcp was then carefully cut to have vertical lanes measuring 6 mm of width spanning the track of the sample. The Ntcp on both sides was then stained (3 mm each side of the lane) in order to locate the proteins of interest. Lane 1 corresponds to the track where 3 μg of TT were loaded, lane 2 had 1.5 μg of TT loaded. Lane 3 corresponds to the stained molecular weight marker. Lane 4, where only loading buffer was run, was used for the preparation of the negative control.
Western blotting clinical study.

Three groups of individuals were compared (patients suffering from MS, neurological controls and normal controls) (see section MM- for details).

(a) Mean concentration of serum IgG (+/- standard deviation (SD)) = \[ \sqrt{\sum x^2 - (\sum x)^2 / n} \] where \( n \) is the number of individuals in each group. \( n - 1 \)

were calculated for each group for comparison.

(b) The number of individuals with autoantibodies against human brain vessels were recorded and the percentage calculated. These results were compared between the 3 groups of individuals using a Chi square test. Yates' correction for continuity was applied when any cell in the calculation contained a value less than 10 [Swinscow T.D.V., 1976].

T cell proliferation studies.

Stimulation was measured in triplicate for each test sample and the count per minute (c.p.m) as well as the disintegration per minute (d.p.m. = c.p.m \times coefficient of efficiency of the machine for the test) were recorded.

Results were calculated as:

1. Mean d.p.m +/- standard error with the corresponding 95% confidence interval.

2. Stimulation indices (SI) which was the mean d.p.m of the test with stimulant / mean d.p.m of test in T cell medium for a classical T cell assay and was the mean d.p.m of the test with stimulant on nitrocellulose paper particles (NCP) / mean d.p.m. of the test with MOCK-NCP (NCP without antigen) for the NTP T cell assay (see section MM-12 for more detail).
MM14: MYELIN PREPARATION:

The myelin preparation method was based on a protocol devised by Dr S. Gillepsie (University of Stirling) [Gillepsie C.S. et al, 1989]:

a/ material:

- Transport medium: sterile Hank's medium pH 7.4 with 250 mM sucrose, 10 mM Hepes, pH 7.4
  3 mM DTT
- 0.25 M sucrose solution: sterile with 10 mM hepes, pH 7.4
  3 mM DTT.
- 0.85 M sucrose solution: sterile with 10 mM Hepes
  3 mM DTT.
- Osmotic shock solution: sterile deionised water
  with 3 mM DTT.
- Storage solution: sterile osmotic shock solution
  with leupeptin 0.5 µg/ml, EDTA 37 µg/ml, pepstatin 1µg/ml
  aprotinin 1µg/ml
- sterile scalpels and forceps.
- sterile petri dishes.
- ultracentrifuge at + 4 °C (Beckman).
- 2 x 50 mls centrifuge tubes, sterile.
- rotating homogeniser.

b/ protocol:

- The brain sample was transported in cold transport medium and keeping all manipulations sterile washed 2 or 3 times with transport medium (the meninges, if present, were removed).
- The sample was weighed and then placed, in a sterile petri dish, in ice cold 0.85 M sucrose solution to make a homogenate of 5 % weight/volume of solution (this
proportion gave the optimum result). The total volume necessary for each preparation was 30 mls.

- The brain was cut into small pieces with a scalpel and a forceps and homogenised with a rotating homogeniser.
- The homogenate was split up into two 50 mls centrifuge tubes and overlaid with the 0.25 M sucrose solution.
- The tubes were centrifuged at 70,000 g for 90 minutes at +4°C.
- Myelin was recovered at the interface between the 0.25 M sucrose solution and the 0.85 M solution was collected and subjected to an osmotic shock by resuspension in 10 volumes in osmotic shock solution and then centrifuged for 30 minutes at 23,000 g, at +4°C.
- The pellet was then subjected to 2 more osmotic shocks.
- The pellet was then frozen at -70°C in storage solution until further analysis.
The technique presented below is a modification of the method described by Brendel et al [Brendel K. et al, 1974]. Different modification of this technique as well as other techniques of purification of brain vessels were investigated and found to be less successful.

**Material:**
- transport medium: Hank's medium
  - with 15 mM glucose
  - 10 mM hepes (pH 7.4)
  - pH adjusted to 7.4 with 1 M NaOH solution
  - autoclaved
- purification medium: transport medium
  - with 5 UI/ml of sterile heparin (Leo Ltd)
  - Penicillin/ streptomycin filtered 100 X solution
  - Fungizone sterile 100 X solution (Gibco)
- PBS
- scalpels and forceps
- petri dishes
- Pasteur pipettes.
- large and small ice polystyrene boxes.
- stand for different mesh sieves with their respective holders.
- mesh sieves (John Staniar & Co, Manchester)
  2 types of sieves are needed: - 210 \( \mu \)m nylon mesh sieve
  - 150 \( \mu \)m nylon mesh sieve
- holders for the sieves: home-made holders were the most practical, for example the bottom of a plastic beaker (beakers of different sizes and circumferences provided the range needed) were cut in order to obtain tubes where a mesh sieve can be taped at one end - the system served as a cylindric filter (see figure MM15-2 for representation). The holders with the sieves were autoclaved in foil at 10 p.s.i.
- beakers for collection of the filtrates through the sieves.
- rotating homogeniser
- homogeniser pestle piston and corresponding glass tubes
  2 types were needed: one loose pestle piston of around 350 \( \mu \)m of aperture and one tight pestle piston of around 200 \( \mu \)m of aperture.
- glass universals
- plastic universal tubes for centrifugation (V bottom).
- inverted microscope and light microscope.
- Eppendorf tubes for storage

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**Figure MM15-2**: Representation of the holders for the sieves.

b/Method: See Figure MM15-1 for general overview of the method. All procedures were carried out in sterile and cold conditions.

- The post mortem brain sample was transported to the laboratory in ice cold transport medium and washed in a petri dish with cold PBS, 3 times.
- The meninges and the surface vessels were stripped off the brain sample and discarded.
- The brain sample (about 10 grammes) was placed in cold purification medium and cut into small pieces of about 2 mm$^3$.
- The pieces were then homogenised in cold purification medium with 20 up-and-down strokes of the loose pestle piston. The first homogenate was homogenised a second
Post mortem brain sample → 2mm³ pieces → Filtration through a 210μm mesh sieve → Collection of the trapped vessels → Filtration through a 150μm mesh sieve → Collection of the trapped vessels → Centrifugation 10 mins. 1000g → VISUALISATION: Inverted microscope, cytocentrifuge slide, G transpeptidase, Factor 8 SDS Page, Polyclonal antisera → Storage

Figure MM15-1: Overview of the brain vessel purification method.
time with 20 up-and-down strokes of the tight piston pestle at 100 circles/minute.

- The homogenate was then filtered through the 210 µm mesh sieve (see Figure MM15-3). The filtrate was kept, homogenised again and filtered - if diluted with the purification medium - through a 150 µm mesh sieve. However, the principal source of purified brain vessels was from the trapped material on the 210 µm sieve mesh. The material was collected with a Pasteur pipette and medium and then homogenised with 20 up-and-down strokes of the tight pestle piston at 100 circles/minute.

- This homogenate was filtered through a 150 µm sieve.
- The trapped brain vessels were collected on the sieve.
- The yield and purity of the preparation were checked by light microscopy.
- The vessels were processed as desired. For antigen studies, the vessels were centrifuged for 10 minutes at 1000g and the pellet stored at -70 °C.

**Figure MM15-3:** Homogenisation and filtration of the brain sample.
The culture of human brain endothelial cells was attempted. The optimal method of growing such a cell line is described below and discussed in the Result and Discussion sections. This method is derived from published work by groups who have claimed to have succeeded in growing human brain endothelial cell lines [Vinters H.V. et al, 1987] [Gerhart D.Z., 1988] and from review on the culture of microvascular endothelium [Pearson J.D., 1990].

a/ Material: - scalpels, forceps, petri dishes, Pasteur pipettes.
- all the material described in the Section on brain vessel preparation (MM15).
- astrocyte conditioned medium from U 138 astrocyte medium [Clapham P.R. et al, 1989] or GO-C-CM conditioned medium [Frame M.C. et al, 1984](European cell culture collection) or from normal astrocytes grown in culture could provide the source of growth factor (see section astrocyte culture).
- Endothelial Cell Growth Factor (E.C.G.F.) (Sigma) Stock solution is 3 mg/ml in water or medium and can be stored at - 20 °C.
- selenium/transferrin/insulin media supplement (Sigma) stock (25µg/25 mg/25mg, respectively) is stored desiccated at -20°C. The stock is diluted with 5ml distilled water (DDW) then with 45 ml DDW. It is left for 1 hour at room temperature. The 50 mls can be diluted to a final concentration (5µg/ml, 5 µg/ml,5ng/ml respectively) into 5 liters of medium. After reconstitution, it should be stored at + 4 °C.
- human brain endothelial cell culture medium: 10 % foetal calf serum (heat inactivated) or human serum 10 % new born calf serum (heat inactivated)
  20 mM Hepes
  Glutamine 4 mM
  Selenium/Transferrin/Insulin
  Heparin 2 IU/ml or 20-40 µg/ml
  ECGF 20 µg/ ml
  40 % ACM.
  in Iscove/F12 Ham's medium (Gibco)
- collagenase (Sigma).
- 25 cm$^3$ and 75cm$^3$ cell culture flasks.

b/ **Method:-** The brain sample was obtained from a temporal lobectomy and transported to the laboratory in cold sterile Hank's buffer, pH 7.4 with Penicillin /Streptomycin and 10 % NBCS.

- The brain was washed 3 times in PBS. The pia matter and the associated vessels were removed with a forceps and the grey and white matter of the brain sample was placed in 'vessels purification medium' (see section on vessels purification).

- The purification of the vessels was done exactly as described in the section "Brain vessels preparation".

- When the vessels were collected from the 150 μm mesh sieve, they were centrifuged at 500 g, 5 minutes) and resuspended in 1ml of warm M199 medium containing 1 mg/ml of sterile collagenase solution.

- The vessels were incubated at 37 °C in collagenase solution for 5 to 15 minutes. The vessels were visualised by microscopy.

- At the end of the incubation the vessels were resuspended in M199 with 10 % NBCS and centrifuged at 500 g for 5 minutes.

- The vessels were resuspended in human brain endothelial cell culture medium and seeded, if possible, 5-10 fragments into a fibronectin coated cell culture petri dishes.
MM17: UMBILICAL CORD ENDOThelial CELL CULTURE.

This technique was provided by Dr Jeremy Pearson (Clinical Research Center, Harrow).

a/ Material: - cannulation: sterile Luer female to Luer male adaptors, product n° 700/180/700; distributor HR Howell. The adaptors were scored with a hot needle, in the middle all around their periphery. This helped to fix the ends of the cord to the adaptor with a string (see figure MM17-1).

![Figure MM17-1: Representation of the adaptor for cannulation of the umbilical cord vein](image)

- Hank's buffer + bicarbonate (pH 7.4)
- PBS
- scalpels, scissors and forceps.
- paper towel, and cling film
- crocodile clips.
- syringes
- collagenase (Sigma)
- culture medium: Medium 199 (Gibco)
  10 % new born calf serum (NBCS)
  10 % foetal calf serum (FCS)
  10 mM Hepses
  2 mM Glutamine
  Penicillin / streptomycin / Fungizone
  4 UI/ml of Heparin
  20 µg/ml of endothelial growth supplement (ECGF).
  ECFG is optional for the first passage.
- 25 cm³ and 75 cm³ cell culture flasks
- 0.2 % (w/v) gelatin (Sigma) in PBS, kept at +4 °C.
- conical centrifuge tube (Falcon).

b/Method:
- The umbilical cord was collected in a sterile container from the maternity ward (cords less than 36 hours old can be used). The cord must be kept in Hank's buffer at + 4 °C.
- In the laboratory, a board platform covered with foil was placed inside a cell culture hood and the preparation of the cell culture was carried out on this board.
- The 2 ends of the cord were cut in order to trim the extremities of the cord. All areas with clamp marks were cut off.
- The 2 ends of the cords were cannulated with the adaptors through the umbilical vein. To do so, all fibrous processes around the 2 extremities of the umbilical vein were dissected out. The adaptors were then securely fixed to the cord with a string which was guided along the scored line (see figure MM17-2).
- The umbilical vein was flushed with warm PBS or Hank's buffer (about 20 mls). All the clots were expelled (when only one end of the cord was cannulated). All leaks were stopped with sterile crocodile clips (see figure MM17-2). The 2 ends-cannulations were connected to 2x10 mls syringes.
Figure MM17-2: Representation of the umbilical cord vein with the 2 cannulated ends connected to the syringes.

- The buffer was then expelled from the cord
- 10 mls of 0.5 mg/collagenase in warm medium 199 + bicarbonate without serum was added with a syringe inside the umbilical vein.
- The cord was wrapped in cling film and left in the incubator at 37 °C. The incubation time was between 5 to 7 minutes (longer increased the contamination with smooth muscle cells).
- The cord was flushed and the contents collected in a conical centrifuge tube. 10 to 20 mls of Hank's buffer were added to the cord and flushed again 3 or 4 times by moving up and down the piston of the 2 syringes. Then the Hank's buffer was added to the centrifuge tube.
- The tube was centrifuged at 250 g for 10 minutes.
- The pellet was washed once with Hank's buffer, centrifuged at 250 g for 10 minutes.
- The pellet was then resuspended in culture medium and incubated in a 25 cm³ cell culture flask which may have...
been previously coated with 0.2% gelatin. The content of one cord were grown in one 25 cm$^3$ cell culture flask.

- Coating of the cell culture flask with gelatin (optional): 0.2% gelatin was prepared in sterile PBS and incubated in the cell culture flask for 30 minutes at 37°C. The cell culture flask was then washed twice in PBS. The gelatin coated flask could be stored about a week at +4°C. Just before culture, the flask was washed twice with warmed PBS.

- When the endothelial cells had reached confluence they were passed with trypsin-EDTA, washed once in Hank’s buffer and split 1 in 3.
MM18: CULTURE OF HUMAN ASTROCYTE.
Reference: Barna and colleagues [Barna BP, 1985].

a/ Material- transport medium:
   RPMI 1640
   10 % NBCS
   Penicillin / streptomycin

- astrocyte cell culture medium:
  RPMI 1640
  10 % FCS
  2 mM glutamine
  Penicillin / streptomycin.

b/ Method - The brain sample was collected at neurosurgery (glial tumor removal or operation for vascular tumor or temporal lobectomy for intractable epilepsy).
- The sample was transported in cold transport medium to the laboratory and placed in a petri dish.
- The brain sample was washed 3 times in PBS, the pia matter and the associated vessels are removed with a forceps.
- The brain sample was placed in astrocyte cell culture medium, minced and then flushed with a syringe through a small gauge needle.
- The pieces were cultured in astrocyte cell culture medium until some cells were seeded at the bottom of the tissue culture dish.
- The floating elements were then discarded and the cells were allowed to reached confluence
- They were split (1 in 2) with trypsin-EDTA after one wash (centrifugation 800g 10 minutes).
MM19 : γ-GLUTAMYL TRANSPEPTIDASE ASSAY.

This method has been described by L.E. DeBault and P.A. Cancilla [DeBault L.E et al, 1980]

a/ Material: - cytocentrifuge slides
   - cytocentrifuge
   - Pasteur pipette, forceps.
   - antigen preparation with negative control available
   - acetone
   - substrate-acceptor-indicator solution:
     0.5 mM g-glutamyl-4-methoxy-b-naphthylamine
     20 mM glycylglycine (Koch-light).
     0.05 % (w/v) Fast Blue BB (Koch-light).
     0.25 % (v/v)dimethyl sulfoxide.
     in 25 mM PO4 buffer saline (pH 7.4)
   - chelator: CuSO4; 0.1 M in 25 mM PO4 buffer saline (pH 7.4).

b/Method: - Cytocentrifuge slides of the test sample with its negative control are prepared e.g for brain vessels a centrifugation of 100 g for 5 minutes is adequate.
   - The slides were air dried overnight at +4 °C.
   - The preparations were fixed with cold acetone for 10 minutes, then rinsed twice with distilled water.
   - They were then incubated with the substrate-acceptor-indicator solution for 90 minutes at 37 °C. The cytocentrifuge preparation, on the slide was covered by the solution; generally 500 µl of the solution was sufficient.
   - After the incubation, the chelator was added for 1 to 2 minutes; this incubation time was checked by inverted microscopy.
   - The reaction was stopped with distilled water.
Purified vessels in chilled Hanks buffer solution with heparin and antibiotics were centrifuged at 1000g for 10 minutes. After change of medium by gentle aspiration the vessels were incubated at 4 °C in 4% glutaraldehyde in phosphate buffer saline overnight. Immediately before preparation of the vessels for SEM, the vessels were dehydrated through increasing concentration of alcohol in PBS : 30 minutes at 50%, 15 minutes at 75%, 15 minutes at 95%, and twice for 15 minutes in absolute alcohol (between each incubation the purified vessels were allowed to sediment and the alcohol solution sucked off). The purified vessels were then allowed to sediment on circular 10 mm microcoverslips (Horwell A.R. Ltd). The preparation was then put into a Samdri critical point dryer with alcohol replaced with liquid CO₂ and dried. It was then fixed with tape on a stup and placed in a EMSCOPE sputter water where it was covered in 10 nanometers of gold and viewed in a JEOL JSM 35 CF scanning electronmicroscope.
MM21: TRANSMISSION ELECTRONMICROSCOPY OF THE MYELIN PREPARATION.

Myelin was prefixed overnight in 2.5% (v/v) glutaraldehyde in PBS at +4°C and then postfixed in 2 ml of 1% (w/v) osmium tetroxide in PBS for 1 hour at room temperature. The myelin was centrifuged at 1000g for 10 minutes and after removal of the osmium tetroxide, the myelin was rinsed in PBS, dehydrated using ascending acetone concentrations (50%, 70%, 90% and 100% (v/v)) for 15 minutes corresponding to each concentration and then fixed overnight in 50% (v/v) araldite (Taab) in 100% acetone on a rotary mixer at room temperature. The araldite-acetone was then replaced with 100% araldite from which all the air had been previously been removed under vacuum and aliquots of the preparation were placed in conical beam capsules (Agar Aids) with the myelin at the base. The resin was then polymerised at 600°C for 24 hours after which thin section of approximately 40 nm were cut using a Huxley ultramicrotome and collected on copper grids (Emscope, 300 nm).

Grids were stained for 5 minutes in Reynolds lead citrate, washed in distilled water for 30 seconds and counterstained in 2% (w/v) aqueous uranyl acetate. The sections were visualised using a Phillips electron microscope (Model EM 301).
MM22: CLINICAL STUDY USING THE WESTERN BLOTTING ASSAY AND MYELIN AND HUMAN BRAIN VESSELS AS ANTIGENS:

a/ The human brain vessels antigen preparation

Human brain vessels were prepared from a single brain < 36 hours after death (see section MM15). The vessels were assessed by light microscopy, electron microscopy (see section MM-20), presence of von Willebrand factor by indirect immunofluorescence (see section MM7) and γ-glutamyl transpeptidase (γG.Tr.Pase) as described in section MM19 and in result section R4.

b/ Human myelin antigen preparation.

Myelin was prepared from a single post mortem brain < 36 hours after death but different from the brain used for the preparation of the human brain vessels. The characterisation and assessment of the preparation are described in sections MM14 and MM21 and in result section R3.

c/ Selection of the MS and the neurological patients and normal controls for the clinical studies.

MS patients and neurological controls were selected by the neurologists and their clinical and biochemical data recorded. The assay was carried out in a blind fashion. When the code was broken, the diagnoses were as follow: MS 45, epilepsy 20, Parkinson's disease 2, myasthenia gravis 2, migraine/headache 5, stroke 2, neuropathy (1 diabetic) 2, unexplained syncope 2; 1 each of: vertigo, carpal tunnel syndrome, hyperventilation, anoxic brain damage, vasculitis, pineal tumor, cervical spondylitis, prolapsed disc, polymyositis, hysteria, cramp, motor neurone disease. Eighteen normal sera from laboratory personnel (department of Biochemistry and Microbiology, St Andrews University) were also tested. The mean age of the MS group was 45 years (range 27-67), the neurological controls 45 years (range 16-84) and the normal controls 28 years (range 22-60). Table MM22-1 summarizes the characteristics of the patients and controls selected for the 'western blotting' clinical study.
<table>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Number</th>
<th>age (range)</th>
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</thead>
<tbody>
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<td>MS</td>
<td>45</td>
<td>45 (27-67)</td>
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<tr>
<td>Normal controls</td>
<td>18</td>
<td>28 (22-60)</td>
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<td>Neurological controls</td>
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<td>45 (16-84)</td>
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Table MM22-1: Characteristics of the patients and controls selected for the western blotting clinical study.
d/ Western blotting assay for the clinical study.

- IgG level was determined for each patient by an immunodotblot technique [Randall R.E.R et al, 1984] using standard controls (ICL scientific) and thus the assay was standardised for the amount of IgG (see Results section R1 for an example).
- Myelin and vessels were denatured in electrophoresis buffer (containing SDS and β-mercaptoethanol), heated at 75°C for 5 minutes and run on SDS-PAGE gel (15 % acrylamide) on a single line made by a one tooth comb. Molecular weight standard markers (BRL, Bethesda Research Laboratory) were run in parallel in order to characterise the vessel proteins (see figure R3-1 for detail of the molecular weight marker). The proteins were then blotted onto nitrocellulose paper for 1 hour at 0.8 mA/mm² using a semidry blotter (LKB). After blotting, the nitrocellulose paper where the antigens had been transferred was cut into 20 vertical and parallel strips and the strips on both sides were stained with naphthalene Black in order to locate the pattern of the antigen (the myelin or the human brain vessels).
- Each strip was incubated with the aliquoted serum (each serum dilution contained 120 μg of IgG per strip in 400 μl of 1% Marvel/PBS; this corresponded to dilutions ranging from 1/25 to 1/60 for the tested sera) for 1 hour, washed and then incubated with the second antibody viz an anti-human-polyvalent immunoglobulins linked to horse radish peroxidase (1/50 in 400 μl of 1% Marvel/PBS) (Sigma). The reaction was visualised by diaminobenzidine as described in section MM10.
MM23: CLINICAL STUDY USING THE "NITROCELLULOSE T CELL ASSAY" WITH MYELIN AND HUMAN BRAIN VESSELS AS ANTIGENS.

a/ Preparation of the antigen preparations (myelin and vessels) for the 'nitrocellulose T cell assay'.
- The same myelin and vessel preparations used for the western blotting clinical study were used for the 'nitrocellulose T cell' assay.
- Eleven different types of myelin antigen nitrocellulose particles and seventeen different types of human brain vessel nitrocellulose particles were prepared as described in section MM12, part B. The protein bands selected from the myelin preparation spanned most of the myelin preparation corresponding to the molecular weight region between 15 to 200 kd and particular attention was taken to include the region corresponding to the myelin basic proteins region (18 to 22 kd) - band 1, the proteolipid protein region (25kd) - band 2 [Cruz T.F. et al, 1985]. The proteins bands selected on the human brain vessels preparation spanned the whole preparation corresponding to the molecular weight region between 10 to around 120 Kd (see figure MM23-1).

b/ Selection of the MS patients, neurological controls and normal controls for the T cell assay.
- MS patients and neurological controls were selected by the neurologists and their clinical and biochemical data recorded. The T cell proliferation assay (section MM11) was carried out in a blind fashion.
- When the code was broken, the diagnoses were as follow. Six MS patients, 4 patients suffering from epilepsy, one patient suffering from diabetes and one suffering from brain damage.
- Six normal controls were also tested and were selected from the laboratory personnel. They were not selected in a blind fashion unlike the neurological patients.

c/ 'Nitrocellulose T cell' assay for the clinical study.
- The assay was carried out in a blind fashion and only one pair of patients (one MS, one neurological control) was tested at each run. The six normal controls were tested independently thereafter.
- For each patient medium alone, PHA, NCP-blank (NCP with blotted denaturing buffer alone, 11 different myelin protein-NCP and 17 different vessel protein NCP were tested. For the last 3 pairs of patients
myelin spotted on NC and electrophoresis buffer-disrupted vessels spotted on NC were also included. NCPs were incubated for 7 days with $2 \times 10^5$ mononuclear cells/200 μl well. Each well was pulsed with 0.5μCi 3H thymidine for the last 16 hours and then harvested and counted.

Figure MM23-1: Blots of brain vessels (A) and myelin (B) samples used for the preparation of the nine myelin protein-NCPs and the eleven vessel protein-NCPs. A/ lane 1: stained molecular weight marker; lane 2: brain vessels (3 mm are stained both sides of the track which has been used for the preparation of the NCPs) - the corresponding bands are located on the right side of the track. B/ lane 1: loading buffer alone (for the preparation of the MOCK-NCPs); lane 2: stained molecular weight marker; lane 3: adenovirus type 2; lane 4: myelin preparation - 9 bands were prepared and are located on the right hand side of the track.
RESULTS

PARAMYXOVIRUS SV5 RELATED CSF STUDIES.

Twenty three CSFs from patients suffering possible or definite multiple sclerosis were supplied by the neurologists in Aberdeen, Dundee and Edinburgh. Great care was taken in the transport of these CSFs which were collected in sterile glass containers. On arrival at the laboratory, they were aliquoted, labelled and stored at -70°C until testing. The CSFs were not frozen and thawed more than once before analysis.

The immunoglobulin concentration was measured in the CSFs by immunodotblot [Randall RE et al, 1985]. Figure R1-1 shows an example of this screening procedure where a known control (from ICL) was used. Table 1, column 2, provides the data for the 23 CSFs. The CSFs were then screened for the presence of oligoclonal bands using the isoelectrofocusing (IEF) technique and silver staining (see Materials and Methods and reference [Goswami K.K.A. et al, 1987]). An example of the silver staining of IEF gels is shown in fig. R1-2a and R1-2b with some of the CSFs tested. The column 3 of table 1 summarises the data for the 23 CSFs.

Concerning the virological investigation, the CSFs were first screened by indirect immunofluorescence using SV5 infected VERO cells. Positive and negative control monoclonal antibodies were readily available in the department [Randall R.E and Young D.F., 1988] and only optimally infected slides were used for this screening procedure. Figure R1- 3a shows a positive reaction with a CSF from an MS patient and figure R1-3b shows a negative reaction. The results are given in column 4 of table 1. CSFs positive for the presence of antibodies to SV5/PF2 were then titrated by the same method using doubling dilutions of the CSFs starting with undiluted sample. The slides were always read by 2 examiners (BES and WCR) in a blind fashion. Two CSFs showed reasonable levels of antibodies to SV5 (i.e titration > 1/400) and were thus selected for further studies. They were first titrated for PF2 antibodies and measles antibodies. Table 2 summarises the data of this study.

The absorption experiments were then carried out on these two CSFs. The principle of the method, described in detail in the Methods section, is based on the fact that immune complexes (the antigen being the whole virus) can be pelleted after high speed
## Table 1: List of the 23 CSFs tested using the immunoabsorption technique.

Column 2: level of IgG (C= in the CSF (mg/dl); S= serum (mg/ml)).
Column 3: presence of oligoclonal bands after isoelectrofocusing and silver staining.
Column 4: presence of antibodies to SV5/PF2 by indirect immunofluorescence.

<table>
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<tr>
<th>SOURCE OF CSF</th>
<th>CODE</th>
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<td>++</td>
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<tr>
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Figure R1-1: Immunodotblot of CSF and sera from MS patients with an anti-human immunoglobulin linked to peroxidase. Two μl of the different samples and their corresponding double dilutions were spotted on the nitrocellulose paper from bottom to the top of the picture. Lanes 1 and 11: double dilutions of the IgG standard starting at 12 mg/dl. Lanes 2 to 10: double dilutions of the CSF (C) or serum (S) of 5 different patients (lane 2: TH (C); lane 3: KK (S); lane 4: KK (C); lane 5: JL (S); lane 6: JL (C); lane 7: DB (S); lane 8: DB (C); lane 9: CG (S); lane 10: CG (C). The CSFs started neat and the sera were diluted 1/100 prior to the test. The estimated levels of IgGs are recorded in table 1 column 2.
Figure R1-2a and 2b: Silver staining of CSFs tested after isoelectrofocusing with some showing oligoclonal bands (see table 1 for the list of the patients). R1-2a/ lane 1: DB; lane 2: EG; lane 3: CG; lane 4: KK; lane 5: DH; lane 6: EM. R1-2b/ lane 1: EM; lane 2: DH; lane 3: JG; lane 4: SP; lane 5: PB; lane 6: WM; lane 7: McRa; lane 8: HG; lane 9: OM.
**Figure R1-3a:** CSF positive for SV5/PF2 antibodies by indirect immunofluorescence on SV5 infected VERO cells. (magnification x 400).

**Figure R1-3b:** CSF negative for SV5/PF2 antibodies by indirect immunofluorescence on SV5 infected VERO cells (magnification x 400).
**ANALYSIS OF THE SELECTED CSF'S**

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**Absorption** with SV5 FLU PBS negative results

**Table 2:** Antiviral titers (anti-SV5, PF2 and anti measles) of the selected CSFs which were positive for antibodies to SV5/PF2 by indirect immunofluorescence (see table 1).
centrifugation in contrast to free antibodies which are retained in the supernatant. If purified virus is mixed with CSFs containing antibodies to this same virus, immune complexes will result from the specific reaction of these antibodies to this virus. Because in this system, there is an excess of antigen, it is likely that, if these antibodies are part of the CSF oligoclonal IgGs, oligoclonal bands corresponding to these antivirus antibodies will not be present in the supernatant of the CSF centrifuged after its incubation with the virus. The technique used to compare the possible difference was the silver staining IEF pattern of the supernatants of different aliquots of a CSF, each incubated with a specific purified virus or with a negative control mixture e.g PBS.

This approach was used with the 2 selected CSFs named CG and TH. A preparation of influenza virus (a gift from the National Institute for Medical Research (NIMR)) was used as a negative control. The SV5 virus was prepared in the department (BES and B. Precious), the purity of the preparation being checked by SDS PAGE (figure R1-4). The respective concentration of the virus preparation was measured by dot-blot assay and the concentration of each virus each in the absorption assay was therefore standardised (1 mg/ml of virus). Five aliquots of each CSF were processed: one aliquot was incubated with purified influenza virus, one with purified SV5 virus, one with PBS, one was not mixed with anything but left overnight at +4 °C and the last CSF aliquot kept at - 70° C overnight. Figures R1-5a and R1-5b show the silver staining IEF pattern of the 5 aliquots of CSF CG and CSF TH after centrifugation. It was clear that the patterns of both CSFs were slightly different between the aliquots but it was not related to the virus itself. For example in Figure R1-5a the patterns between aliquots did not differ greatly apart from the last track which corresponded to the CSF alone incubated at +40 °C overnight. In Figure R1-5b, however, the oligoclonal bands have almost disappeared from CSF-TH which was incubated with PBS (track3) or with influenza (track 4). Figure R1-6 shows another negative absorption experiment (TH repeated, EM and JL CSFs)

Different factors could have induced this effect. As previously established in the department, the technique appeared to be reproducible and little difference was seen between different tracks of the same sample especially when they were silver stained in parallel (Dr K. Goswami, personal communication). It is noticeable
Figure R1-4: SDS-PAGE minigel of the purified SV5 preparation used for the immunoabsorption experiments. Five µls were loaded on the gel as shown on the photograph. The proteins of the virus are named on the right side of the track.
Figure R1-5a: Immunoabsorption with the CSF CG (see table 1 and 2). From left to right track 1: incubated with purified influenza virus overnight. Track 2: incubated overnight- nothing added. Track 3: incubated with SV5 overnight. Track 4: incubated with PBS overnight. Track 5: not incubated overnight- nothing added.
**Figure R1-5b:** Immunoabsorption with the CSF TH (see table 1 and 2). From left to right; track 1: not incubated overnight; track 2: incubated overnight with SV5; track 4 incubated overnight with influenza virus; track 5: incubated overnight.
**Figure R1-6:** Immunoabsorption experiments with 3 different CSFs from patients with MS (JL, TH, EM). JL's CSF and TH's CSF are CSFs with antibodies specific for SV5 (see table 1) EM's is a CSF with no antibodies specific for SV5 (see table 1). Tracks 1,2,8,9,10: CSF from JL- track 1 and 10 incubated with PBS; track 2 and 8 incubated with measles virus; track 9 incubated with SV5. Tracks 3 and 4: CSF from TH - track 3 incubated with SV5; track 4 incubated with PBS. Tracks 5, 6, 7: CSF from EM - track 5 incubated with measles virus; track 6 incubated with SV5; track 7 incubated with PBS.
that in the IEF of the TH and CG aliquots the tracks which were on the edges were better stained than the other tracks. This is not the case with other IEF gels of CSFs and thus the position of the tracks was not a likely explanation for the discrepancy seen between the aliquots of TH. It was then hypothesised that the ionic strength, phosphate content or pH of the CSF mixed with different mixtures of virus or PBS may have influenced the properties of the relatively cathodic oligoclonal IgGs. It is therefore possible that non-immune reactions e.g absorption or precipitation may have been the cause of the original observations [Goswami K.K.A. et al, 1987] indicating that a large proportion of oligoclonal bands in CSFs of some MS patients were reacting to the virus SV5.

To test this hypothesis the following experiment was set up: aliquots (95 µl) of a CSF from a patient (EM) were incubated with 5 µl of phosphate buffer solutions (phosphate 50 mM in 0.1 M NaCl) of different pH (track1, pH 6; track 2, pH 7; track 3, pH 8). Another aliquot was incubated with phosphate buffer of pH 7 but with high salt content (0.25 M NaCl)(track 4). The last track (number 5) corresponded to the CSF alone. The aliquots were centrifuged as previously described and their supernatants run in IEF and silver stained. The result of this experiment is shown in figure R1-7. The staining of the oligoclonal bands in the supernatant run on track 4 was less than in the other tracks. Track 5 was the aliquot which had not been diluted and the pattern was not different from track 1, 2 or 3. This correlated with previous observations made in the department that the dilution effect due the addition of 5 µl of buffer to the 95 µl of CSF was not responsible for the difference of staining seen between aliquots which had been incubated with a virus preparation or PBS and a CSF aliquot which has not been diluted. The overall conclusion of this experiment was that the high salt content of aliquot 4 may have induced a precipitation of some oligoclonal bands.

To corroborate this conclusion, another experiment was designed: aliquots of CSF-EM were incubated overnight with virus, PBS or on its own. They were then centrifuged and the pellet of each of these aliquots was dissolved in denaturing mixture (see Methods section, SDS-PAGE electrophoresis) and a sample run on a SDS PAGE minigel which was then silver stained. The gel is shown in Figure R1-8. Track 1 was pellet of aliquot not diluted, track 2, 3 and 4 corresponded to aliquots incubated with influenza virus, SV5.
Figure R1-7: Isoelectrofocusing of the CSF EM after changing pH and salt content. From left to right track 1,2,3: phosphate buffer 50 mM in 0.1 M NaCl, track 1: pH 6, track 2: pH 7, track 3: pH 8. Track 4: pH 7 in phosphate buffer 50 mM in 0.25 NaCl. Track 5: CSF incubated overnight. Track 6: CSF not incubated overnight.

Figure R1-8: SDS-PAGE minigel of the pellets after centrifugation of the CSF EM incubated overnight with: track 1 CSF alone, track 2 incubated with influenza virus, track 3 incubated with SV5, track 4 incubated with PBS. Heavy (H) and light (L) chains from immunoglobulins are marked.
virus or PBS respectively. The conclusion of this experiment was that the immunoglobulins (heavy and light chain on an SDS gel) appeared in the pellet when the CSF was incubated with virus (track 2 and 3) but they were also precipitated, especially the heavy chain, when the CSF was incubated with PBS. This effect was not seen with the undiluted CSF aliquot.

In conclusion it was suggested from the above experiments that changes in ionic strength, phosphate content and pH may have been responsible for some of the differences observed in the original observation even though phosphate buffer and control antigens were run in parallel at that time.

**ASSESSMENT OF THE TECHNIQUES USED IN THE STUDIES OF THE POSSIBLE INVOLVEMENT OF SV5 IN MS: THE IMMUNOABSORPTION, IMMUNOPRINTING AND THE IMMUNOBLOTTING OF CSF.**

The aim of this investigation was to compare these 3 techniques which were used by different research groups in the study of the possible involvement of SV5 in MS. The immunoabsorption technique was currently in use in the Department at St Andrews, the imprint immuno-fixation method was used by Dr Vandvick in Norway and the immunoblotting method was used by Dr McLean in London (see the Discussion section).

The imprint immuno-fixation method depends on the ability of the oligoclonal bands displayed after concentration of the CSF and isoelectrofocusing on polyacrylamide gels to be transferred sequentially to agarose gels containing different virus antigens (virus specific antibodies) or to agarose gel without anything (detection of oligoclonal pattern). The antibodies which attach are then detected by 125 I labelled anti-human IgG. The virus antigens are generally crude extracts of infected cells and controls are derived from extracts of uninfected cells (see Material and Methods section).

The principle of the immunoblotting technique is only slightly different: unconcentrated CSFs are separated by isoelectrofocusing agarose gels and then blotted onto nitrocellulose coated with or without antigens (to detect virus specific antibodies or to assess the oligoclonal pattern respectively) (see also Material and Methods).
The principle of the third method, the immunoabsorption technique, has been described in the previous section and also in the Materials and Methods section. The interpretation of the results is based on the disappearance of bands corresponding to oligoclonal IgG which have been absorbed by a specific antigen. These bands are visualised after isoelectrofocusing by silver staining. The sensitivity of the technique therefore depends greatly on the sensitivity of the silver staining methods for CSF oligoclonal IgG.

Two parameters were assessed: the specificity and the sensitivity of these 3 methods. To achieve this aim, a defined system was chosen: monoclonal antibodies specific for parainfluenza 2 and SV5 virus were available in the Department. Monoclonal antibodies 161 and 173/2 (specific for SV5) and monoclonal 77/4 (specific for PF2) are protein A binding-monoclonal antibodies [Randall R.E. et al, 1987] and thus were purified using a protein A column. Figure R2-1 shows the monoclonal antibodies characterised by SDS PAGE after purification. The protein content of the purified preparation of monoclonal antibodies were then measured by dot blot (see Method section MM10').

![Figure R2-1: SDS-PAGE of the viruses and of the monoclonal antibodies, after purification on protein A column, used to assess the different CSF techniques described in section R2, stained with coomassie blue. From left to right, tracks 1 and 2 show the viral preparation used: track 1, influenza virus, track 2, SV5 virus. Monoclonal antibody 161 (track 3) is specific for SV5, 173/2 (track 4) is specific for SV5, 77/4 (track 6) is specific for PF2. Track 5 shows a purified preparation of human immunoglobulins (not used for the work presented in this thesis).](image-url)
a) Sensitivity of the techniques:

1. Silver staining method:
   Different concentrations (0.5, 0.1, 0.05, 0.025, 0.0125 mg/ml) of the monoclonal antibody 173/2 were examined using an isoelectrofocusing gels and the gel was silver stained. Figure R2-2 shows the resulting silver stained gel indicating that the sensitivity of the method was 0.05 mg/ml using a 10 µl sample.

Figure R2-2: Silver staining on electrofocusing polyacrylamide gel of different concentrations of 10 µl samples (from track 1 to track 5: 0.5, 0.1, 0.05, 0.025, 0.0125 mg/ml) of the monoclonal antibody 173/2.
2. Imprint-immunofixation method:

Different concentrations of the monoclonal antibodies 77/4 (2, 1, 0.5 mg/ml) were examined on an isoelectrofocusing gel alongside the 2 mg/ml dilution of the monoclonal antibody 173/2 (The monoclonal 77/4 is specific for the parainfluenza virus PF2 and 173/2 is specific for SV5 and does not cross react with PF2 [Randall R.E., 1987]). After isoelectrofocusing of these 2 monoclonal antibodies, the gel was put in contact with the agarose gel which contained PF2 virus infected VERO cells for 5 minutes. The agarose gel was then incubated with Iodine 125 labelled protein A. Figure R2-3a shows the separating gels after transfer and Figure R2-3b shows the transfer pattern onto PF-2 -containing agarose gel. The sensitivity of the technique appears to be 1 mg/ml with the monoclonal antibody 77/4 (anti PF-2). However, the specificity of the technique is questionable as 173/2 (not specific for PF-2) also transferred at the concentration of 2mg/ml. No lower concentrations of 173/2 were included.

Figure R2-3a: Sensitivity and specificity of the imprint immunofixation method. Isoelectrofocused agarose gel stained with coomassie blue after transfer by the immunoprinting method of different concentrations of the monoclonal antibody 77/4 (track 1 to 3: 2, 1, 0.5 mg/ml loaded on the gel) and 2 mg/ml dilution of the monoclonal antibody 173/2.
Figure R2-3b: Sensitivity and specificity of the imprint immunofixation method: PF2-containing agarose gel used for the transfer of the monoclonal antibodies isoelectrofocused in figure R2-3a is shown. This gel was overlaid on the gel shown in figure R2-3a and the monoclonal antibodies allowed to transfer between the 2 gels. The transferred monoclonal antibodies were visualised by protein A labelled with iodine 125 (see material and methods).
3. Immunoblotting method.

a/ Sensitivity for non specific transfer of antibody:

This technique is used to detect the presence of oligoclonal IgGs in clinical samples. Two alternatives are available: the IgGs are either transferred on uncoated nitrocellulose paper and the transferred IgGs are then detected by an anti IgG and then by the second antibody conjugated to peroxidase. The second possibility is that the first antibody (the anti IgG) is coated on to the nitrocellulose paper before the transfer of the IgGs. Different concentrations (0.5, 0.1, 0.02, 0.025, 0.0125, 0.0063, 0.0031 mg/ml) of the monoclonal antibody 173/4 were thus transferred either to uncoated nitrocellulose paper (figure R2-4a) or to anti-mouse IgG coated nitrocellulose paper (figure R2-4b). In both cases, the sensitivity is < 0.003 mg/ml with perhaps a better pattern at the lower concentration for the anti-mouse coated IgG nitrocellulose paper.

**Figure R2-4:** Sensitivity of the immunoblotting method. Different concentrations (from right to left: 0.5 (lane 1), 0.1 (lane 2), 0.02 (lane 3), 0.025 (lane 4), 0.0125 (lane 5), 0.0063 (lane 6), 0.0031 (lane 7) mg/ml) of the monoclonal antibody 173/4 (specific for SV5) were transferred to nitrocellulose paper which was

**R2-4a:** uncoated

**R2-4b:** coated with an anti mouse IgG.
b/ Sensitivity for detection of virus specific antibody.

In this experiment, the nitrocellulose paper was coated with SV5 infected cells. Different concentrations (0.5, 0.05, 0.02, 0.01, 0.005 mg/ml) of the monoclonal antibody 173/2 were transferred to this SV5 coated membrane. The transferred monoclonal antibody was then visualised by an anti-mouse IgG linked to horse radish peroxidase. Figure R2-5 (section A) indicates the result of the experiment and the sensitivity of the technique indicates 0.005 mg/ml similar to that for the non specific transfer of IgG.

Figure R2-5: Section A: Sensitivity of the immunoblotting method with virus coated nitrocellulose paper. The nitrocellulose paper was coated with SV5 infected cells. Different concentrations (0.5(a), 0.05(b), 0.02(c), 0.01(d), 0.005(e) mg/ml) of the monoclonal antibody 173/2 (specific for SV5) were transferred to this SV5 coated nitrocellulose paper. The concentrations are decreasing from right to left. The transferred monoclonal antibody was then visualised by an anti-mouse IgG linked to peroxidase.

Section B and C: Specificity of the immunoblotting method. Different dilutions (from right to left) of the monoclonal antibody 161, specific for SV5 (0.25(a), 0.05(b), 0.02(c), 0.01(d), 0.005(e)) [B] and different concentrations of the monoclonal antibody 77/4, specific for PF2, (1(a), 0.5(b), 0.25(c), 0.05(d), 0.02(e) mg/ml) [C] were transferred onto SV5 infected cells coated nitrocellulose paper after isoelectrofocusing and then visualised by an anti mouse IgG labelled with peroxidase.
b) The specificity of the techniques:

1. The immunoabsorption technique.
   The experiments which assessed specificity of this technique have been described in the section R1 (CSF studies in relation to the paramyxovirus SV5).

2. The imprint immuno-fixation method.
   This technique was not explored much further. One experiment was carried out however to assess if the overlay contact time of the 2 gels influenced specificity. Monoclonal 173/2 and 77/4 were run at the concentration of 1 mg/ml. the gel to gel contact time was 30 seconds, 2 min, and 5 minutes. It appears that at 30 seconds, the technique is specific whereas when the contact time is longer non specific reactions occur.

3. The immunoblotting technique.
   In the first experiment, different dilutions of 3 monoclonal antibodies were isoelectrofocused in parallel: 173/2 (0.25, 0.05, 0.02, 0.01, 0.005 mg/ml) a monoclonal antibody specific for SV5, 161 (0.25, 0.05, 0.02, 0.01, 0.005 mg/ml) a monoclonal antibody specific for SV5 and 77/4 (1, 0.5, 0.25, 0.05, 0.02 mg/ml) a monoclonal antibody specific for PF-2. The gel was then overlaid with nitrocellulose coated with SV5 infected cells. Figure R2-5 (section C) shows the result of this experiment indicating that at 1 mg/ml the monoclonal antibody 77/4 is apparently transferred and detected thus the specificity of the technique is called into question at this concentration.
   In the next experiment, other antigens were tested in a similar way with different monoclonal antibodies. Three types of antigen coated nitrocellulose papers were tested viz SV5 coated nitrocellulose paper, measles virus (Mv) coated nitrocellulose paper and adenovirus (Ad) coated nitrocellulose paper (see Material and Methods section); the SV5 purification has been described in the previous section (see Figure R1-4); the adenovirus was purified by Ian Leith in the laboratory and the measles virus was purified by Bernie Precious in the laboratory. Four different monoclonal antibodies were run at the same concentration (0.02 mg/ml): 173/2(SV5 specific), 161 (SV5 specific) [Randall R.E. et al, 1987] ,

164
B 10 (Mv specific) [Russell W.C. et al, 1983] and 13G4G9 (Ad specific against the fiber type 2) [Russell W.C., personal communication]. Figure R2-6 shows the result of this experiment. On the SV5 coated nitrocellulose paper, 173/2, 161 and B10 were transferred and detected, on the Mv coated nitrocellulose paper the four monoclonal antibodies were transferred, and, on the adenovirus coated nitrocellulose paper only the 13G4G9 (Ad specific) was transferred. Therefore, in this experiment, at the concentration tested and with the monoclonal antibodies tested, only the adenovirus system was specific.

Figure R2-6: Specificity of the immunoblotting technique. Three types of antigen coated nitrocellulose paper were tested viz SV5 coated nitrocellulose paper (Ntcp) [A], measles virus (Mv) coated Ntcp [B] and adenovirus Ntcp [C]. Four different monoclonal antibodies were run at the same concentration (0.02 mg/ml). From left to right: B10 (Mv specific), 13G4G9 (adenovirus specific), 161 (SV5 specific) and 173/2 (SV5 specific).
A similar result, was obtained a second time with a SV5 coated nitrocellulose paper when B10 (Mv specific), 77/4 (a PF-2 specific monoclonal antibody) and 173/4 (SV5 specific) were tested. All 3 monoclonal antibodies reacted with the SV5 coated nitrocellulose paper. B10 and 173/2 were transferred onto the nitrocellulose paper coated with SV5 infected cells. The Mv - coated nitrocellulose paper was more difficult to interpret but B10 and 173/2 appeared to have transferred.

Figure R2-7: Specificity of the immunoblotting technique. Monoclonal antibodies B10 (Mv specific) [1], 77/4 (adenovirus specific) [2] and 173/2 (SV5 specific) [3] were transferred on SV5 coated nitrocellulose paper [A], on SV5 infected cells coated nitrocellulose paper [B] and on measles coated nitrocellulose paper [C].
SUMMARY AND CONCLUSION OF R1 AND R2 SECTIONS:

Oligoclonal IgGs specific for SV5 were not found in the series of 25 multiple sclerosis patients tested using the immunoprecipitation technique. Consequently, a series of experiments assessed this technique in an attempt to explain a previous report of SV5 specific oligoclonal IgGs detected in the CSF of MS patients. It was found that the modulation of ionic strength, phosphate content and pH of the buffer used in the immunoprecipitation assay could lead to non immunological precipitation of the relatively cathodic oligoclonal IgGs and thus to false positive results in some cases. This result stimulated an assessment of two other CSF techniques (immunoblotting and imprint-immunofixation) which had also been used for looking at the presence of SV5 specific antibodies in multiple sclerosis CSFs. The immunoblotting technique was extremely difficult to control and was prone to non specific reaction. On the other hand, the imprint-immunofixation technique was easier to control but again the technique showed a narrow window of specificity.

In view of these relatively negative results and in the light of current theories of the MS process it was decided to look at a related aspect of the aetiopathogenesis of MS i.e potential target autoantigens in the induction of MS following a widespread theory that a possible autoimmune reaction leading to demyelination could be induced, in MS patients, by an infectious agent. In accordance with the histopathological features of multiple sclerosis, the possible autoantigens which were considered at the time of this study were human myelin and human brain vessels. In order to test this hypothesis, techniques were developed to purify and characterise antigens for the cellular and humoral immunological studies. The next 3 sections describe the purification and characterisation of human brain myelin, the purification and characterisation of brain vessels and the attempts to establish a human brain endothelial cell line in culture.
PURIFICATION OF MYELIN FROM HUMAN BRAINS:

Myelin was prepared from a single post mortem brain less than 36 hours after death. The brain was provided by the courtesy of Dr J. Anderson (Department of Pathology, Ninewells Hospital, Dundee).

The sucrose gradient method for the preparation of myelin is described in detail in Material and Methods. The yield of the preparation was very satisfactory: from a brain sample of 1.5 gm, 7 mls of myelin preparation were obtained with a protein concentration of 1.5 mg/ml as measured by dot-blot.

The polypeptide pattern of the preparation on SDS PAGE (15% acrylamide) was in agreement with the published pattern of myelin [Wray W.T. et al, 1981] (see Figure R3-1). The purity of the preparation was then checked by transmission electronmicroscopy with the help of J. Mackie (Department of Biology and Preclinical medicine, St Andrews University).

Figure R3-1: SDS PAGE (15% acrylamide) of the myelin preparation (track 1) and of the molecular weight marker (track 2) (from the bottom: 14 kd, 18 kd, 29 kd, 43 kd, 68 kd, 97 kd, 200 kd).
Multiple sections were examined and it was concluded that the preparation was substantially of myelin. Figure R3-2, R3-3, R3-4 show increasing magnification of the preparation.

Furthermore, the preparation was recognised using the Western Blotting technique by a rabbit polyclonal antisera to rat-myelin prepared by Dr E Blair (Department of Biochemistry, Leeds University); this antisera cross reacted with human myelin and recognised most of the proteins of the myelin sheath (E. Blair, personal communication, unpublished data); see Figure R8-2.

Figure R3-2: Transmission electronmicroscopy of the myelin preparation at low magnification (x 4200).
Figure R3-3: Transmission electronmicroscopy of the myelin preparation at medium magnification (x 25 000).

Figure R3-4: Transmission electronmicroscopy of the myelin preparation at high magnification (x 200 000).
PURIFICATION OF BRAIN VESSELS FROM BOVINE AND HUMAN BRAINS:

There are a number of techniques which describe the purification of brain vessels from animal brains (rodent, canine and bovine) and from human post mortem brains. They are based on different principles which are often combined: 1) enzymatic digestion of the brain by 'dispase', 2) mechanical homogenisation of the brain, 3) separation of the components of the brain by different gradient systems e.g. dextran, sucrose or percoll, 4) filtration through nylon sieves of different mesh sizes, 5) use of glass beads to trap the vessels (see the Discussion section for references). These different procedures were assessed on the basis of yield and purity using bovine brains obtained from the local slaughterhouse and then on samples of human post mortem brains. The post mortem samples were provided by pathologists from Edinburgh (Dr Lessell, Dr MacIntyre and Dr Bell) and from Dundee (Dr Anderson).

Comparison between the methods was done solely with an inverted microscope; photographic facilities were not available with this system and only description and characterisation of the brain vessels purified by the method of choice will be shown. When the other methods were assessed in the department, some techniques (the use of glass beads and the use of dextran and percoll gradient after dispase digestion) were found totally unsuccessful and others (e.g. the use of sucrose gradients after homogenisation) were found to be of limited success. The outline of these unsuccessful methods are presented in figure R4-1. One protocol was found to give very satisfactory results and thus the exploration of the other techniques was abandoned.

This successful brain vessel purification method was the combination of 2 techniques: homogenisation of the brain followed by filtration through nylon sieves of different mesh sizes (described in detail in Materials and Methods). It gave reproducible results with bovine brain and human samples. At the end of the 2 cycles of homogenisation and filtration, the purity of the brain vessel preparation was checked by inverted microscopy and if it was not satisfactory, an additional cycle of homogenisation followed by filtration through another 150 µm nylon mesh sieve was found to improve the purity of the preparation but at the cost of the yield. It was also found that it was possible to increase greatly the proportion of the small vessels (less than 12 µm of diameter) in the preparation.
Figure R4-1: outline of the protocols of the experiments set up to investigate the purification of brain vessels.
when the preparation was filtered through a 150 μm nylon mesh sieve and when the filtrate was then collected over a 40 μm nylon mesh sieve. This modification of the protocol was not utilised in this thesis but its potential is evident for the study of the structure and the composition of vessels of different sizes.

When the preparation was judged to be satisfactory, the vessels were collected and the purity of the preparation was assessed by light microscopy on a cytocentrifuged sample stained with toluidine blue. The preparations normally contained more than 95% of brain vessels. Figure R4-2 shows such a preparation of bovine brain vessels (400 μl were cytocentrifuged; about 10 mls of the same preparation were obtained from 10 grammes of brain). Vessels of different sizes, branched and unbranched, were present i.e capillaries and small venules as well as small arterioles. Figure R4-3 shows, at higher magnification, some of the small vessels stained with toluidine blue and the nuclei of the endothelial cells or the pericytes are very well delineated. The position of the nuclei seemed to rotate about one third of a turn along the course of the vessels. Other figures illustrate some of the scanning electronmicroscopical features of fixed brain vessels from a human post mortem brain 5 days after death: figures R4-4 and R4-5, capillaries; fig R4-6 lumen of the vessel and nucleus (arrow) of a pericyte, fig R4-7 shows the extracellular matrix of a small brain arteriole.

The vessels were characterised by the presence of vW factor (antibody from Dako) using immunofluorescence and a penetration buffer during incubation of the antibody with the vessels in order to allow this antibody to reach intracellular antigens (Fig R4-8). They also showed reactivity for gamma glutamyl transpeptidase (see method section) which is an enzyme apparently specific for brain endothelial cells (Fig R4-9a) [DeBault L.E., 1980]. HELA cells were used as a negative control for gamma glutamyl transpeptidase (Fig R4-9b). The SDS-PAGE pattern of the human brain vessels (Fig R4-10) was compared to the pattern published by one other group [Pardridge W.M. et al, 1985] and it was similar. Some of the proteins of brain vessels are already been characterised to some extent e.g the low molecular weight proteins are mainly histones [Pardridge W.M. et al,1989] and it appears that a 46 kd protein is a blood-brain barrier (BBB) specific protein [Pardridge W.M. et al, 1986] (see discussion for a more complete review).
Figure R4-2: Cytocentrifuged preparation of bovine brain vessels stained with toluidine blue (x 100).
Figure R4-3: Nuclei of the endothelial cells and of the pericytes of purified brain vessels stained with toluidine blue (x 400).

Figure R4-8: Human brain vessels positive for von Willebrand factor after treatment with penetration buffer (x 450).
Figure R4-4: Scanning electronmicroscopy of human brain capillaries isolated from a post mortem brain (x 450) (bar = 100 µm).

Figure R4-5: Scanning electronmicroscopy of human brain capillaries isolated from a post mortem brain (x 900) (bar= 10 µm).
Figure R4-6: Lumen of one of the human brain vessels and nucleus (arrow) of a putative pericyte (x 15000).

Figure R4-7: Extracellular matrix of a small human brain arteriole (x 2800).
Figure R4-9a: Human brain vessel positive for $\gamma$-glutamyl transpeptidase (x 600).

Figure R4-9b: HELA cells negative for $\gamma$-glutamyl transpeptidase (x 600).
Figure R4-10: SDS-PAGE of the human brain vessels preparation (track 1). A molecular weight marker is run in parallel in track 2. The corresponding molecular weights of the protein markers are (from the bottom): 14 kd, 18 kd, 29 kd, 43 kd, 68 kd, 97 kd, 200 kd.
ATTEMPTS TO ESTABLISH HUMAN BRAIN ENDOTHELIAL CELLS IN CULTURE:

Attempts at the establishment of rat or human brain endothelial cell lines were unsuccessful. It was hoped that these cell lines would be available for the T cell and the 'western blotting' studies (see corresponding sections). This approach was not explored further and purified brain vessels were used as antigens in the clinical studies and because these clinical studies had a tight schedule, there was not enough time or immediate purpose to explore further the growth of brain endothelial cell lines.

A. Culture of astrocyte cell lines

It has been demonstrated that most of the brain-specific properties of the endothelial cells in the brain (e.g. the possession of tight junctions and an enzyme system like gamma-glutamyl transpeptidase (γ-GTPase)) are induced by the local environment [Golstein G.W., 1988] especially by the astrocyte cells [Janzer R.C. et al, 1987]. Secondly, it is well known that the brain specific properties of endothelial cells can be lost after a few passages in culture [Gerhart D.Z. et al, 1988] and that brain endothelial cells can regain some of their brain specific characteristics when they are co-cultured with astrocytes or astrocyte-conditioned medium [Tao-Cheng J.H. et al, 1987] [Arthur F.E. et al, 1987] [Debault L.E. et al, 1980]. It was therefore anticipated that it would be easier to culture a human brain endothelial cell line if this cell line was cultured in astrocyte-conditioned medium. Thus the first goal was to grow human astrocyte cells in culture.

Two brain samples were available during this time, one was from a resection for a brain aneurysm (the sample, kindly provided by Mr Varma, was macroscopically normal) and the second came from resection of a high grade glioma grade III (kindly provided by Mr Wittle). The protocol for growing astrocyte cells was the method described by Bama and colleagues [Bama B.P. et al, 1985]: the cells were cultured on a petri dish after mincing the brain sample (see Method section). In both cases, cells grew and were confluent after 7 to 10 days and were passaged at least 20 times. One culture was named N.GLIA and came from the normal biopsy and the other was named GLIO and came from the glioma sample. In the meantime, antibodies specific for the glial lineage cell - Glial
Fibrillary Antigen Protein (GFAP) - were obtained (one rabbit polyclonal anti-GFAP from Dako and one monoclonal anti-GFAP from Sera Lab). Cells that had been passaged at least 3 times were aliquoted and frozen in liquid nitrogen. With a DAPI stain (see Materials and Methods, section MM7), none of the 2 cell lines appeared to be infected with mycoplasma (see figure R5-1) but they were also 100% negative for GFAP (see figure R5-2). The 2 cell lines (GLIO and N.GLIA) were tested for other markers and were both positive for an anti-factor VIII/vW - an endothelial specific marker - provided by the Scottish Antibody Production Unit (SAPU). Figure R5-3 shows GLIO cells positive for SAPU anti-factor VIII/vW. At higher magnification perinuclear granules positive for the marker were also visible (figure R5-4). This appearance was possibly due to Weibel-Palade bodies which are thought to be storage and/or processing organelles for the von Willebrand (vW) protein [Wagner D.D. et al, 1982]. However the culture morphology was not that of an endothelial cell line which has a typical cobblestone morphology at confluence. Figure R5-5 shows that GLIO cells produced long appendages as with glial type cell line and when subconfluent the culture had a "hill and valley"-like morphology more in keeping with a smooth muscle cell line (see Figure R5-4). The SAPU-anti factor VIII/vW marker was also positive on VERO cells and it therefore appears that the SAPU polyclonal anti-factor VIII/vW can cross react with unknown cellular proteins non specific for the endothelial cell lineage (Dr Peters; BTS, Edinburgh, personal communication). A monoclonal antibody Eswf/2, which is specific for von Willebrand factor was also utilised (kindly provided by Dr Peters) and it was found that GLIO and N.GLIA did not express this specific marker.

In conclusion, the 2 cell lines (GLIO and N.GLIA), which were thought to be astrocytic cell lines, did not express GFAP. They did not express vW factor specific for the endothelial cell lineage, and in culture they had a morphology which could be related to the astrocyte lineage or to smooth muscle lineage. At the time, there was no reliable specific marker for smooth muscle cells.

As an alternative to 'home-made' astrocyte cells, 2 cell lines of the astrocytic lineage were obtained from external sources. The first cell line, kindly provided by Professor Weiss (ICR, London), was U138 and had been shown to be susceptible to HIV despite its lack of
Figure R5-1: Absence of mycoplasma contamination of cell line GLIO as visualised by DAPI stain (magnification x 800) (N.GLIA cell line did not show any infection by mycoplasma either).

Figure R5-2: GLIO cell line negative for GFAP. Some cells are mitotic (--->) (N.GLIA was also negative for GFAP (magnification x 400).
Figure R5-3: GLIO cell line positive for VIII/vW antigen by immunofluorescence using the SAPU antibody (magnification x 600).

Figure R5-4: Perinuclear granules visible with the SAPU anti-factor VIII/vW in the GLIO cell line (magnification x 1200).
Figure R5-5: Long appendages of GLIO cell line stained with the SAPU anti factor VIII/vW (magnification x 600)
expression of the CD4 receptor [Clapham P.R., 1989]. The second cell line, G-CCM, was obtained from the European Cell Culture Organisation and was originally produced by I. Fresney's group in Glasgow [Frame M.C. et al, 1984]. Both cell lines were originally said to express 100% GFAP. However, when tested in our laboratory, only 10% of the cells expressed this marker. Again, this phenomenon of limited expression of GFAP by astrocyte cells has been reported [Frame M.C. et al, 1984]. Figure R5-6 and R5-7 show the GFAP reactivity with G-CCM cells and U-138 respectively. Conditioned culture media from these 2 cell lines were collected, centrifuged to eliminate cell debris and stored at -20°C for further use.

Figure R5-6a: G-CCM cell line stained with anti-GFAP (Dako).

Figure R5-6b: U 138 cell line stained with anti-GFAP (Dako).
B. Human umbilical cord endothelial cells.

As already discussed, characterisation of any brain cell line in culture is hampered by the fact that the cell line can stop expressing markers specific for the lineage of origin. This is well documented with both types of cell lines dealt with in this chapter - endothelial and glial cell lineage. It was therefore important to establish well characterised endothelial cell lines which are known to express consistently endothelial lineage markers in order to assess the different antibody markers available and possibly to use this characterised cell line as standard. The astrocyte cell lines have already been described above.

A well established source of endothelial cells is the umbilical cord and with advice from Dr Jeremy Pearson (Clinical Research Center, Harrow) a method for culturing human umbilical vein endothelial cells (HUVEC) was devised (see Material and Methods Section). Umbilical cords were obtained from consenting patients in the maternity hospital at Kirkcaldy.

The culture of HUVEC was successful, and during this time multiple markers specific for the endothelial lineage were collected from different sources and tested on HUVEC and on negative control cell lines (HELA and VERO cells). Table R5-1 gives a list of all the markers tested and the results observed on known cell lines.
Table R5.1: List of specific markers tested on HUVEC and other known cell lines using the immunofluorescence method.

<table>
<thead>
<tr>
<th>MARKER</th>
<th>TYPE</th>
<th>SOURCE</th>
<th>COMMENTS</th>
<th>RESULTS - immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Willebrand factor</td>
<td>EsWf Mab</td>
<td>Dr Peppers - gift (BTS Edinburgh)</td>
<td>endothelial specific</td>
<td>+ HUVEC, purified brain vessels, brain section</td>
</tr>
<tr>
<td></td>
<td>Polyclonal</td>
<td>Dako</td>
<td></td>
<td>- HELA, VERO</td>
</tr>
<tr>
<td>UEA-1</td>
<td>lectin</td>
<td>Vector lab</td>
<td>endothelial specific</td>
<td>+ HUVEC, - HELA, VERO</td>
</tr>
<tr>
<td>Angiotensin converting enzyme</td>
<td>rabbit polyclonal</td>
<td>Dr Turner - gift (Leeds)</td>
<td>fairly specific for endothelial cells</td>
<td>+ HUVEC, - HELA, VERO</td>
</tr>
<tr>
<td>Gamma glutamyl transpeptidase</td>
<td>enzyme assay</td>
<td>Koch-Light</td>
<td>brain specific if endothelial</td>
<td>+ purified brain vessels - HELA</td>
</tr>
<tr>
<td>M14 Mab</td>
<td>Mab</td>
<td>SAPU - gift</td>
<td>macrophage specific</td>
<td>+ monocytes - HELA</td>
</tr>
<tr>
<td>EMB II Mab</td>
<td>Mab</td>
<td>Dako - gift</td>
<td>macrophage and neuroglial</td>
<td>- HELA</td>
</tr>
<tr>
<td>GFAP Mab</td>
<td>polyclonal</td>
<td>Seralab</td>
<td>glial specific</td>
<td>+ human brain section, U138 - HUVEC</td>
</tr>
<tr>
<td>Isoactin Mab</td>
<td>Mab</td>
<td>Sigma</td>
<td>smooth muscle specific</td>
<td>+ human brain section - HUVEC</td>
</tr>
<tr>
<td>HLA Class I W32 Mab</td>
<td>polyclonal</td>
<td>Seralab - gift</td>
<td></td>
<td>+ HUVEC</td>
</tr>
<tr>
<td>Class II DP</td>
<td></td>
<td>Becton Dickinson - gift</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td></td>
<td>SAPU - gift</td>
<td>B cell specific</td>
<td>- HUVEC, + PBL</td>
</tr>
<tr>
<td>CD4, CD8, CD3</td>
<td></td>
<td>SAPU - gift</td>
<td>T cell specific</td>
<td></td>
</tr>
</tbody>
</table>

HUVEC = human umbilical vein endothelial cells, PBL = peripheral blood lymphocytes
The frequency of these experiments was greatly influenced by the availability of normal human brain samples from the neurosurgery departments. The only source of such a sample for a human brain endothelial cell line was temporal lobes obtained at lobectomy for intractable temporal lobe epilepsy. This type of neurosurgery is not frequent (roughly 40 cases a year in Great Britain) and 2 such samples were available during the time course of this thesis.

The optimal method for growing human brain endothelial cell lines in culture is described in detail in the Material and Methods section. However some parts of this method needed to be refined and changed. The overall principle of the method was to purify brain vessels from a brain sample taken at surgery and to treat the vessels with collagenase in order to break parts of the structure of the vessels so that endothelial cells migrated outside these vessels. The vessels in culture should stick to the culture petri-dish and then endothelial cells should migrate from these vessels and form a confluent monolayer which can be trypsinised and expanded.

The first human brain sample was processed and purified brain vessels were seeded on culture petri dishes without treatment with collagenase. After a week of culture, the purified vessels were floating but none of the vessels had adhered to the culture plate. It was then thought that the collagenase treatment would make the vessels more sticky as the extracellular matrix -site of protein involved with adhesion processes - was likely to be exposed by the collagenase treatment. Just before obtaining the second human brain sample, the collagenase treatment was standardised with purified rat brain vessels. The brain vessels were purified, treated with collagenase for different lengths of time and a change in the structure of the vessels visualised by inverted microscopy. During the first minutes of treatment with collagenase, the structure of the vessels did not change. However after 5 minutes the architecture of the vessels opened up and the cell nuclei (from pericytes or endothelial cells) protruded to a greater extent than in the intact structure. After about 10 minutes at 37 °C (1 mg/ml of collagenase) the vessels disintegrated. It was thus concluded that a collagenase treatment of 7 minutes was optimal. Unfortunately, despite the collagenase treatment the rat vessels did not stick to the culture petri
dish. The human brain sample arrived before the method with the rat vessels was further refined. The only modification that was introduced for the second human brain sample was that the culture petri dishes were coated with gelatin as an attachment factor (fibronectin or other attachment factors were not available in the department at the time) as it had been reported that the presence of attachment factors on the culture dishes was an important factor [Shivers R.R. et al, 1988]. However, the collagenase treated vessels did not stick to the petri dish even after 5 days in culture.

It is clear from the present limited experience of growing human brain endothelial cell lines in culture and from the small number of published papers that no simple method will work and that multiple factors are critical for the success of such methods [Pearson J., 1990].
INTRODUCTION OF THE T CELL PROLIFERATION STUDIES:

After preparation, the antigens - human brain vessels and human myelin - were used in a T cell proliferation and a humoral assay to assess immune response from multiple sclerosis patients and controls to support the 'autoimmune theory' of multiple sclerosis. A T cell response in MS patients has been sought in response to very few myelin antigens apart from myelin basic protein and it is therefore pertinent to attempt to investigate the possible immune response against other myelin antigens as well as the antigens of the blood brain barrier. It was thus decided to separate the proteins of both myelin and brain vessels and to test each of these proteins in the T cell assay. In order to do so, it was decided to use the 'nitrocellulose T cell assay' which has the potential to avoid difficult purification of proteins. The nitrocellulose T cell technique utilises separation of antigens by SDS-PAGE followed by blotting onto nitrocellulose paper (NCP). The appropriate polypeptide antigen band can then be cut out, dissolved in DMSO and reprecipitated as microparticles of antigen-NCP which can be used as antigen in a standard proliferation T cell assay.

In the following chapter standardisation of the 'NTP T cell' assay is described.
R6: T CELL PROLIFERATION STUDIES:

Abbreviations used in this section (see text):
NTP T cell assay: 'nitrocellulose T cell' assay.
NCP: nitrocellulose particles.
Ntcp: nitrocellulose paper.
Ag-NCP: Antigen-NCP.
Neg (Ag)-NCP: Negative control NCP with irrelevant antigen.
Mock-NCP: Buffer alone-NCP.
PBMCs: peripheral blood mononuclear cells.
FCS: Foetal calf serum
PHA: Phytohemagglutinin
PPD: Purified protein derivative (from Mycobacterium tuberculosis).

Standardisation of the 'NTP T cell assay'.

The first aim of the T cell study was to standardise and optimise the 'nitrocellulose T cell' assay ('NTP T cell' assay). First, the conditions of a standard T cell proliferation assay were assessed in order to compare them with those of a 'NTP T cell' assay. Both techniques were standardised on the same basis that each test sample was incubated in triplicate with 2.10^5 peripheral blood mononuclear cells (PBMCs)/well in a final volume of 200 µl/well. The PBMCs were cultured for a variable period of time (3 days - 5 days with mitogens and 5 to 7 days with soluble antigens or antigen-NCP (Ag-NCP)) and pulsed with 0.5 µCi of ^3H thymidine per well for the last 16 hours of culture. For practical reasons of availability and also of continuity for the comparisons, the PBMCs of one individual (BES) were used as standard in the first instance.

In relation to a standard T cell proliferation assay, mitogenic stimulation was assessed with phytohemagglutinin (PHA) and antigenic stimulation was assessed with different antigens (purified protein derivative (PPD), M. tuberculosis, tetanus toxoid, tetanus toxin, adenovirus, influenza virus). These antigens were chosen and tested for different reasons. Some were available in the department (adenovirus). This antigen preparation as well as influenza were taken as good examples of viral antigens likely to be recognised by normal PBMCs. The other antigens were gifts and were known to be good 'recall' antigens for T cell proliferation assay e.g tetanus toxin or PPD.
However these antigens were only available in the department over a period of time (either because they were gifts or because they had to be prepared). This delay in obtaining a battery of antigens made the assessment of the 'NTP T cell' assay more difficult and longer to undertake than was expected. A chronological summary of this investigation is presented below.

For each set of experiments, appropriate negative controls were prepared depending on the antigen tested and the T cell proliferation assay used (classical T cell assay or 'NTP T cell' assay). Concerning the 'NTP T cell' assay, a simple nomenclature will be followed in order to describe the different nitrocellulose particles (NCPs) prepared (nitrocellulose paper will be called Ntcp). Negative controls were either prepared with irrelevant antigens blotted or spotted on Ntcp and will be called Neg (Ag) -NCP where Ag refers to the antigen used. A second type of negative control - more frequently used in this report - was prepared by spotting or blotting buffer alone on Ntcp and these negative controls NCP will be named MOCK-NCP. The corresponding buffer for these latter NCP was chosen in relation to the experiment performed and will be described for each experiment. The negative control for the blotting experiment was prepared with denaturing buffer alone blotted on Ntcp. In general the negative control for an experiment was prepared in parallel on the same SDS-PAGE gel as the antigens to be tested. This was prepared from a track where electrophoresis buffer alone was run and then blotted on Ntcp as the antigen (see Figure MM12-6 pg 121 for an example).

a/ Influenza virus antigens (gift Mr A. Douglas, NIMR)
First influenza antigens were used but did not induce a proliferation in either a normal T cell assay or the 'NTP T cell' assay. Figure R6-1a shows the SDS PAGE of the virus preparation and Figure R6-1b shows the blot which was used for preparing the NCP related to this antigen preparation. Two major bands are visible on the SDS PAGE gel as well as minor bands, the top major band, F1, corresponded to the viral proteins HA1, NP and NA and the lower band, F2, corresponds to HA2 [Murphy B.R. et al, 1990]. The negative control (Neg (Ag)-NCP ) used in this experiment was normal human serum blotted on Ntcp from which bands corresponding to the molecular weight region of heavy and light chain were cut out; another negative control used was MOCK-NCP.
Figure R6-1a: SDS-PAGE of the purified influenza virus preparation (X 31 strain) used for preparing the FLU-NCP used for the standardisation of the 'NTP T cell' assay. The virus preparation was run on the right track number 2 (the corresponding viral proteins are discussed in the text). Track 1 (left) shows the molecular weight markers starting from the lower band 14 kd, 18 kd, 29 kd, 43 kd, 68 kd and 97 kd.
Figure R6-1b (A): Blot used for the preparation of the FLU-NCP and the MOCK-NCP. The influenza preparation shown in figure R6-1a was run on SDS PAGE using a single comb tooth and then transferred onto the blot shown above. The 2 edges of the blot were stained with naphthalene black and the 2 main bands of the virus preparation located. The central part of the blot, which was only washed with PBS and not stained, was cut in order to select only the 2 viral bands (hatched area) for the purpose of the experiment FLU1 (middle band) and FLU2 (lower band)(the corresponding viral proteins are discussed in the text). An additional band was cut in the high molecular weight region (the top hatched band) to provide the negative control, MOCK-NCP.

Figure R6-1c(B): Second blot used for the preparation of new FLU-NCP using the same virus preparation as shown in figure R6-1a. The negative control, MOCK-NCP, was prepared in parallel from a different blot where electrophoresis had been carried out in the absence of antigen.
Figure R6-2a shows the negative results of the proliferation assay; PHA mitogenic stimulation was satisfactory but none of the antigens-NCPs induced a significant stimulation index over the controls. This experiment was repeated with the same negative results (see Figure R6-2b) using the same system but with newly prepared influenza-NCP particles (see figure R6-1c). The quantity of proteins (about 0.05 μg/ml) used in the T cell proliferation assay in the well corresponded to the quantity used in already published work [Palacios-Boix A.A., 1988] [Abou-Zeid C et al, 1987]. At this point it was not known if the conditions of a T cell proliferation were right for the soluble viral protein or if the control was not immune to the strain (X 31) of influenza virus used to prepare the FLU-NCPs. One colleague (BCP) performed a haemagglutination assay using different strain of influenza virus and BES' serum in order to evaluate the antibody response against different influenza virus. It was shown that BES had antibody against X 31. Antibodies to influenza strain X 31 positive at 1/128; antibodies to influenza strain AJAP 1967 positive at > 1/64; antibodies to influenza strain C positive at < 1/64 and antibodies to influenza strain APR8 1944 (H1 negative) negative. Therefore no definitive conclusion could be drawn in relation to the 'NTP T cell' assay (later on, it was shown that soluble virus X 31 (control not included in experiment described in fig R6-2a and R6-2b) induced a proliferative response with BES' PBMCs. It was therefore decided to test a different antigen which was likely to be positive for inducing a proliferative response with the control's PBMCs.
Figure R6-2a: Result of the T cell proliferation (d.p.m) with FLU1-NCP, FLU2-NCP and MOCK-NCP prepared as shown in figure R6-1b. Proliferations of mononuclear cells incubated with medium alone and with PHA (1 μg/ml) are also shown.

Figure R6-2b: The same experiment as that described in figure R6-2a was repeated with the different NCP prepared as described in figure R6-1c.
b) Purified protein derivative (PPD):

As the normal control (BES) had been vaccinated against tuberculosis, purified protein derivative (PPD) was used as one of the antigens to be tested by this technique. And when it was received in the department (gift from Wellcome), it induced a proliferation with the control's PBMCs. Figure R6-3 shows the result of the proliferative response with soluble PPD and with other antigens tested in the same experiment.

![Graph showing T cell proliferation (d.p.m.) with soluble PPD (50 and 10 μg/ml) to assess that the PPD preparation induced a T cell proliferation with BES' mononuclear cells. PHA 1 μg/ml was also included in the experiment.](image-url)
When the proliferative capacity of the PPD preparation was established, an attempt to prepare the PPD-NCPs was made but the PPD could not be transferred onto Ntcp by electroblotting. This phenomenon is not understood despite an experiment undertaken to explain it. The concentration of the PPD preparation was assessed by dot blot and was 1 mg/ml. As PPD has a low molecular weight of between 9.5 kd to 11 kd, the PPD preparation was run on a 20% SDS PAGE gel and electroblotted onto Ntcp. In this experiment 2 nitrocellulose sheets were placed on the anodic side of the system and one nitrocellulose sheet was placed on the cathodic side of the gel. The sheets were stained with naphthalene black after electroblotting (see Figure R6-4 c, d and e) and the SDS-PAGE gel, after transfer, was stained with coomassie blue (Figure R6-4 a). PPD did not appear on any of the nitrocellulose papers after staining although the PPD seemed to have been transferred properly. PPD was stained by naphthalene black when spotted on Ntcp or when stained on an SDS PAGE (Figure R6-4 b). In conclusion the inability to transfer PPD on Ntcp by electroblotting made any attempt to test this antigen in a 'NTP T cell' assay impossible.
Figure R6-4: Experiment designed to investigate the transfer of PPD on nitrocellulose paper. PPD was run on different tracks of an SDS-PAGE gel. From left to right:
A: track 1: molecular weight marker; Track 2 and 3: PPD stained with coomassie blue.
B: tracks 4 and 5 stained with naphthalene black. The other side of the SDS PAGE gel was transferred onto nitrocellulose paper.
C: gel stained with coomassie blue after transfer of the PPD.
D: first nitrocellulose paper on the cathodic side stained with naphthalene black after electrotransfer.
E: a second nitrocellulose paper was put in contact on the cathodic side of the previous one and also stained with naphthalene black after electrotransfer. Another nitrocellulose paper was also applied on the other side of the SDS PAGE (anodic side) and was also stained with naphthalene black but did not show any stained PPD (not shown).
In the meantime, although the conditions of the T cell assay were not changed and the same batches of T cell medium components used, high background was recorded in the proliferative response of the control's PBMCs alone. A synopsis of the background over time (5 proliferation assays experiments were done between the 10/3/89 and 16/6/89) using the PBMCs from the same person (BES) and the same conditions of culture (5 days) is recorded in Figure R6-5. It was found that the sole agent responsible for this high background was the foetal calf serum (FCS). However, 2 types of media which were available in the laboratory were also tested: RPMI 1640 liquid medium purchased directly from the GIBCO company and RPMI 1640 prepared in the laboratory from the powder purchased from GIBCO company. The latter gave high background whereas the former did not (see Figure R6-6a). Secondly, the effect of mercaptoethanol was tested using mitogen stimulation and it was found that the stimulation indexes with mercaptoethanol included in the culture medium were much higher than when mercaptoethanol was omitted (see Figure R6-6c). Thereafter human AB serum was used in the T cell assays and mercaptoethanol was included in the culture medium.

![Figure R6-5: Increase of the background proliferation count (c.p.m.) over time with BES' PBMCs cultured for a total of 5 days with medium alone. The medium used in all the experiments contained 10% of foetal calf serum from GIBCO.](image-url)
Figure R6-6a: Effect of different sera and media on the proliferation (d.p.m) [A] of BES' PBMC which were cultured for a total of 5 days with medium alone and on the stimulation index with PHA (1 μg/ml) [B].

A.B.C: the T cell medium was made with RPMI 1640 purchased as a liquid form from GIBCO and β-mercaptoethanol was included in medium.  
A: 10% human AB+ (BTS).  
B: 10% FCS (GIBCO).  
C: 10% FCS (Sera Lab).  
D: The T cell medium was made up with RPMI 1640 prepared in the laboratory from a stock powder purchased from GIBCO. The culture medium contained 10% FCS from Sera Lab and β-mercaptoethanol (PHA stimulation with this medium was not tested).
Figure R6-6b: Effect of β-mercaptoethanol on the proliferation of BES' PBMC to different concentrations of PHA which were cultured for a total of 3 days. SI (mean d.p.m with PHA/mean d.p.m with medium alone) is shown for all the test samples.
c) Mycobacterium (M.) tuberculosis (gift from Dr A. Colston NIMR)

M. tuberculosis as soluble antigen induced high proliferative response of the control's PBMCs (see Figure R6-8c) This preparation was successfully transferred onto Ntcp by electroblotting but when used in the 'NTP T cell' assay, it did not induce any proliferation of the control's mononuclear cells. These experiments are illustrated in Figure R6-8 a which shows the SDS-PAGE minigel of the mycobacterium preparation used in the experiment and Figure R6-8 b shows the Ntcp blot used for preparing the 7 different NCPs which spanned over the low and medium molecular weight regions which are likely to contain recall antigens in most individual vaccinated with BCG (Dr Palacios-Boix, personal communication). Figure R6-8c shows the result of the proliferation assay using the NCPs. A lot of effort was thus spent to pinpoint the stage(s) of the procedure which could explain the failure of the technique - the type of nitrocellulose paper (3 types were tested), the batch of DMSO, the carbonate buffer, the washing procedure, the disruption mixture used to dissolve the protein, the T cell medium - but to no avail: changing one of these factors did not help in achieving a proliferative response (data not shown).

Figure R6-8a: SDS-PAGE of the mycobacterium Tuberculosis preparation (used in the proliferation assay reported in figure R6-7) stained with coomassie blue - first track (left). The second track represents the molecular weight marker (right).
**Figure R6-8b:** Nitrocellulose blot of the MT preparation. The blot was used to prepare the 7 mycobacterium-NCPs as labelled on the picture. A different blot with electrophoresis buffer transferred was used to prepared the MOCK-NCPs.

**Figure R6-8c:** Proliferation (d.p.m.) of BES' PBMCs incubated 7 days with mycobacterium tuberculosis and with the 7 different MT-NCPs prepared as shown in figure R6-6b.
After all these negative results and attempts, the 'NTP T cell' assay was discussed with a research worker (Dr Steve Lee, Birmingham [Lee S et al, 1989]) familiar with this technique, and, his advice proved to be useful. Other people had been consulted since the start of the investigation into the 'NTP T cell' assay including Dr A Rees (Hammersmith hospital) and Dr Rook (Middlesex hospital) (see [Abou-Zeid C. et al, 1987]) - who also had great experience in this technique. Numerous possibilities which could explain these negative results were considered with Dr Lee and were then tested in the following experiments.

It was first thought that the NCPs which had been processed in DMSO could be toxic to the PBMCs. Mycobacterium tuberculosis was incubated with previously prepared NCPs to assess if the latter could inhibit the proliferative response induced by M. tuberculosis antigen. This was not the case (see Figure R6-9).

**Figure R6-9:** lack of inhibition by the MOCK-NCPs, used in the experiment described in figure R6-8b, upon the T cell proliferation of BES' PBMC after 7 days incubation with mycobacterium tuberculosis.
The other possibility was that the antigens had lost their antigenicity during the preparation of the NCPs (e.g. because of the DMSO treatment) or that the antigen-NCPs were not able to be processed by the antigen presenting cells present in the wells during the T cell proliferation assay. The latter alternative could have been explained by the possibility that too large a size of the NCPs were obtained. It was thus decided that the preparation of the NCPs - especially the reprecipitation step - had to be refined. Reprecipitation of the particles dissolved in DMSO was achieved, under control condition, by delivering the carbonate buffer at a constant speed of 0.8 ml/min using a peristaltic pump with the nitrocellulose dissolved in DMSO stirred on an electric stirrer, at constant and reproducible speed (see Material and Methods section MM12, figure MM12-4 pg 117). After precipitation, the particles washing was done in the cold. The precipitation of the particles was apparently much more efficient. This became evident when the suspension of the particles was monitored after precipitation: the NCPs stayed in suspension for a longer period of time instead of sedimenting quickly at the bottom of the Eppendorf tubes. The size of the NCPs was very small and they were visualised by scanning electron microscopy. They measured in average less than 1 μm (see Figure R6-10 a, b, c).
**Figure R6-10a:** Scanning electronmicroscopy of an antigen presenting cell (BES') incubated with mycobacterium-NCP. Two lymphocytes can be seen on the cell and the mycobacterium-NCP (arrow); (white bar shown corresponds to 1 μm).
Figure R6-10b: A different antigen presenting cell (APC) is photographed. The NCP are shown (arrow) and it is thought that platelets are also present on the APC (*); white bar = 10 μm.

Figure R6-10 c: High magnification of mycobacterium- NCPs (arrow) on the APC (white bar shown corresponds to 1 μm). At least 4 lymphocytes (L) can be seen.
However, it was also realised that both, the amount of Ntcp in relation to the amount of DMSO, was critical for the optimal precipitation of the Ntcp. These two parameters were interdependent in producing an optimal curve of precipitation - the result of an experiment demonstrating this is shown in figure R6-11: 6mm x 2 mm Ntcp and 6mm x 4 mm Ntcp were dissolved in different amounts of DMSO (150 µl, 250 µl, 500 µl) and then reprecipitated with carbonate buffer delivered with a peristaltic pump at 0.8 mls per minute. Precipitation was scored as degree of cloudiness and time of naturally occurred sedimentation of the NCPs was also registered. Then each aliquot was centrifuged on a minifuge to visualise the volume of the pellet obtained. The optimum quantity of DMSO for 6 mm x 2 mm Ntcp was 250 µl and the optimum concentration of DMSO for 6 mm x 4 mm Ntcp was 500 µl.

The spotting technique: (see Material and Methods, section MM12, page 112)

Following other workers' advice (Dr G.Rook and E. Filley [Abou-Zeid C. et al, 1987][Filley E. et al, 1989]), it was also realised that the spotting technique could give invaluable data on the standardisation of the T cell technique with less steps involved in the preparation of the NCP. Mycobacterium spotted onto Ntcp was tested successfully (see Figure R6-12, track A to F): ten µg of M.tuberculosis was spotted on 20 mm² Ntcp and after treatment with DMSO and reprecipitation and washing the MT-NCP were resuspended in 1 ml of medium, aliquoted and stored at - 70°C. The optimum concentration/well was 10 µl (0.2 mm², 0.1 µg of this solution (see below).

As the next step, experiments were carried out to standardise the method further by analysing the different factors which could influence a proliferative response (see figure R6-12). These were tested at least twice to assert the reproducibility of the method. In particular, dose dilution effect of the NCP-antigen upon the T cell proliferation assay were studied (see also the blotting technique (below)). It was found that unlike a standard proliferation assay where only the concentration of soluble antigen (Ag) per well (usually 1-100 µg/ml) is critical, 2 factors have to be taken into consideration for the 'NTP T cell' assay - the quantity of antigen/mm² of Ntcp and the quantity of Ag-NCPs that is incubated into each well during the T cell proliferation assay. From the result
of the present study, and, comparing with the literature [Abou-Zeid C. et al, 1987] [Lamb J.R. et al, 1988] [Lee S.P. et al, 1989], it was concluded that the optimum concentration of Ag and corresponding quantity of Ntcp per 200 μl well was 0.01-0.1 μg and 0.1 -0.4 mm² respectively. These parameters were calculated as a function of the concentration of Ag loaded or spotted at the beginning of the procedure and do not take into consideration the possible loss of antigen during the procedure e.g during the washing of the NCPs. These parameters can be further optimised when one considers the type of antigen used in the assay. For example, for a complex antigenic preparation like mycobacterium tuberculosis spotted on Ntcp, one should choose the higher range of the optimum concentration of antigen (0.1- 0.05 μg /well) whereas for an antigen which represents a single protein band (e.g 155 kd TT transferred on a blot) a much lower concentration of antigen is more appropriate (0.02-0.01 μg/well) (see figure R6-14).

Other factors related to the preparation of the NCPs were studied e.g the sonication of the particles prior to incubation for the T cell assay and the washing of the Ntcp prior to dissolution by DMSO. It was found that sonication of the particles did not improve the stimulation ( figure R6-12, tracks G) and that washing the Ntcp with detergent (after the spotting of protein) could induce loss of protein from the Ntcp (figure R6-12, tracks H to J). Thereafter another experiment was set up to determine the importance of washing of the Ntcp during the blotting technique and will be described below under the blotting technique section.

The time course of the T cell proliferation assay using NCP-antigen particles was also assessed (see figure R6-13) using soluble mycobacterium tuberculosis and mycobacterium tuberculosis-NCPs as antigens. PHA (1 μg/ml) was also tested for comparison with mitogenic T cell stimulation. It was found that the optimum culture time was 6 to 7 days for the 'NTP T cell assay' with unsatisfactory stimulation index at 5 days whereas it was 5 to 7 days with a classical T cell proliferation assay .
Figure R6-11: Interrelationship between the quantity of DMSO and the surface of nitrocellulose paper on the precipitation of the NCP by the carbonate buffer. Two different sizes of NCP (6 mm x 2 mm; 6 mm x 4 mm) were incubated with different quantities of DMSO (150, 250, 500 µls). The precipitation was graded by 3 different methods: 1. visually: after precipitation the solution became uniformly cloudy; however if there was no precipitation, the solution remained clear. 2. The time of the natural sedimentation of the nitrocellulose particles: the time registered was when the pellet began to appear at the bottom of the Eppendorfs. 3. The size of the pellet after sedimentation was graded visually.
Figure R6-12: T cell proliferation with different Mycobacterium tuberculosis (MT)-NCPs prepared by spotting the MT on Ntcp before DMSO treatment. The samples were incubated for 7 days.

A: medium alone
B: M.T. : 2 μg/ml.
C: NTP-Blank, control of E (2 mm² of Ntcp/well)
D: NTP-Blank, control of F (0.2 mm² of Ntcp/well)
E: M.T.-NCP (2 mm² of Ntcp/well; 1 μg of M.T. /well)
F: M.T.-NCP, 1/10 dilution of E.
G: sonicated M.T.- NCP (same concentration as F)
H: M.T.-NCP (the M.T. was boiled dissolved in electrophoresis loading buffer (disruption mixture) but the Ntcp was not washed before treatment with DMSO, same concentration as F/well.
I: NTP was processed in the same manner as H but the NTP was washed with PBS+ Np 40.
J: Same as I but the quantity of M.T. spotted on the Ntcp was twice as in the case of I.
Figure R6-13: Time course of the T cell proliferative response with soluble mycobacterium tuberculosis (MT) (2 µg/ml), MT-NCPs (0.2 mm², 0.1 µg of MT/well), and PHA (1 µg/ml).

The stimulation indices for each samples are shown:
- Stimulation index of MT (d.p.m sample/ d.p.m medium alone)
- Stimulation index of PHA as above.
- Stimulation index of MT-NCP (d.p.m MT-NCP/d.p.m NTP-Blank)

The numbers in bracket shows the corresponding d.p.m background for each sample.
The blotting technique (see Material and Methods section MM12, pg 119)

When the spotting technique was thought to be reproducible the blotting technique was tested with proteins electroblotted on Ntcp. To achieve this 2 types of system were used: 1. Tetanus Toxin (TT) electroblotted on Ntcp, 2. Adenovirus proteins electroblotted on Ntcp. The protocol of the blotting technique is basically the same as the protocol for the spotting technique. A home-made comb was devised in order to optimise the loading of the proteins on the SDS PAGE minigel. The representation of the measurement of the comb is shown in Figure MM-12 page 119 (Material and Methods section) and fitted a minigel apparatus. Figure MM12-6, page 121 (Material and Methods section) shows the blot prepared with purified Tetanus Toxin (gift from Dr Colin Watts, Dundee). This blot was used to prepare the 3 different TT-NCPs and the MOCK-NCPs. Denatured TT shows 3 bands (155 kd, 100 kd, 55 kd) as seen in figure MM12-6 (and Dr C. Watts, Dundee, personal communication). Six μl (3 μg) of the stock TT solution (0.5 mg/ml) were loaded on a gel and half of the track was processed, thus 1.5 μg of TT. Bands of 6 mm x 212 mm²) were cut out for each protein band (155, 100 and 55 kd), thus the 155 kd band (roughly half of the total weight of TT as seen on the gel page 121) was processed at 0.75 μg / 12 mm² and resuspended in 500 μl of T cell medium and stored aliquoted at -70°C. The final concentration of the 155 kd band-NCP was 8.33 μl /well (0.2 mm² Ntcp,0.012 μg/ 200 μl well (x4, /ml)). Using this comb system, the system was well standardised when assessed on a 'NTP T cell assay' (see Figure R6-14).
Figure R6-14: Proliferative response of different TT-NCPs (TT= tetanus toxin). The samples were incubated for 7 days.
A: medium alone.
B: TT (1 μg/ml)
C: TT 155 kd-NCPs (0.2mm², 0.012μg/well)
D: TT 155 kd-NCPs (0.2mm², 0.022μg/well)
E: TT 155 kd-NCPs 1/4 dilution of C.
F: TT 155 kd-NCPs 1/10 dilution of C.
G: TT 100 kd-NCPs (0.2mm², 0.006 μg/well).
H: TT 55 kd-NCPs (0.2mm², 0.006 μg/well).
I: NTP-MOCK, control of C, D, G, H (0.2mm² of Ntcp).
The other system used was the adenovirus type 2 (Ad-2) (prepared in the laboratory by Ian Leith [Russell W.C. et al, 1967 and 1982]) and the nitrocellulose blot used to prepare the different adenovirus proteins-NCPs is shown in figure R6-15a. Six main adenovirus proteins were visible on the blots (from high molecular to low molecular weights (H (hexon), P (penton), F (fibre), V, VI and VII) and NCPS were prepared for the 6 proteins. These Ad2 proteins-NCPs were tested in a T cell proliferation assay and H, F and VI induced a proliferation when incubated with BES' PBMC with F producing the most proliferation. However in this particular experiment the background counts were higher than in the previously T cell proliferation experiments (medium alone and MOCK-NCPs) (see figure R6-16).

Figure R6-15a: Blot used for the preparation of the Ad2 proteins-NCPs. Track A shows the transferred Ad2 proteins with their corresponding names. Track B: molecular weight marker. Track C: Ad2-proteins blotted, the width of the track was originally 12 mm (same as track A but a 6 mm lane was cut out in the middle in order to prepare the Ad2 proteins-NCPs corresponding to H, P, F, V, VI and VII.
**Figure R6-15b**: T cell proliferation (d.p.m) experiment using the Ad2 proteins-NCPs prepared as shown in figure R6-15. The T cell assay was done over 7 days.
Some experiments were then undertaken in order to standardise and to assess the blotting and the spotting technique further. One experiment established that the storage of the Ag-NCPs does not alter their antigenic potential. This was demonstrated with TT 155kd-NCPs stored 2 months at -70°C. These particles were compared with newly prepared TT 155kd-NCPs which had only been stored 24 hours at -70°C. Both NCPs induced comparable T cell proliferation of BES’ PBMC (see figure R6-17)

**Figure R6-17:** T cell proliferation (d.p.m) experiment in order to assess the influence of the storage of NCPs at -70°C for a period of 2 months. The T cell proliferation induced by TT 155-NCPs freshly prepared (new) was compared with the T cell proliferation induced by TT 155-NCPs which had been prepared 2 months previously and stored at -70°C until the T cell proliferation assay.
One other experiment assessed the influence of the washing of Ntcp after electrotransfer (see in Figure R6-18), just before the treatment of the Ntcp with DMSO during the preparation of the NCPs. Doubling dilutions of a bovine serum albumin (BSA) solution (1mg/dl) was either run on a SDS-PAGE and electrotransferred, or, after the electrotransfer spotted on the same Ntcp. A third technique [Randall RE et al, 1985] was also analysed: Aliquots of BSA solution were double diluted in the wells of a Terasaki plate and then put into contact for 1 hour with a nitrocellulose paper which was sandwiched between 2 plates (the top Terasaki plates contained the BSA dilutions). The nitrocellulose paper which had been in contact with the different dilutions of BSA was then either not washed, washed in PBS, washed with 0.3% tween 20 in PBS and then with PBS alone or washed with 0.05% Np 40 in PBS and then in PBS alone. It was found that BSA directly spotted on Ntcp as well as BSA spotted on Ntcp by the immunodot blot method were washed away if detergent (Np 40 or Tween 20) was included in the washing buffer. However, when the BSA was electroblotted, it was not significantly washed away with detergent although it appears that there was some loss of proteins when compared to the washing without detergent. It was therefore decided, for the 'NTP T cell' assay that the proteins spotted- or electroblotted-Ntcp should be washed briefly in PBS without detergent before treatment with DMSO (spotting and electroblotting could therefore be compared).
Figure R6-18: Effect of the washing on the retention of proteins electroblotted or spotted on Ntcp (see text for description of the experiment). In the central part of the photo, the nitrocellulose paper used for the electrotransfer of the double dilutions of BSA is shown (top part of each central nitrocellulose paper). On the same Ntcp, double dilutions of BSA were also spotted after transfer (lower part of each Ntcp). On the sides, double dilutions of BSA were transferred to a different Ntcp using the immunodotblot method with Terasaki plates. The different Ntcps were subject to different washing procedures. A. no washing. B. washing with PBS. C. washing with 0.3% Tween 20 in PBS. D. washing with 0.05 % Np 40 in PBS. The different Ntcps were then stained with naphthalene black and compared for the retention of proteins.
CONCLUSION OF THE STANDARDISATION OF THE T CELL ASSAY AND INTRODUCTION TO THE CLINICAL STUDY.

The standardisation of the 'NTP T cell' assay has been achieved with 3 different antigenic systems: mycobacterium tuberculosis, tetanus toxin and adenovirus 2. The critical factor for the success and reproducibility of the technique was the size of the antigen nitrocellulose particles. The particles had to be very small to be presented to lymphocytes in the T cell assay. This was achieved by resuspending the nitrocellulose paper particles, previously dissolved in DMSO, in a bicarbonate buffer at constant and slow speed using a peristaltic pump. When reproducibility was achieved it was found that the technique was sensitive in the well controlled systems investigated.

The next step of the project was to undertake the clinical study (the NTP T cell assay and western blotting assay) with the antigens previously purified (myelin and brain vessels) with patients suffering from multiple sclerosis compared with neurological controls and normal controls. These investigations are described in the last results sections.
R7- CLINICAL STUDY USING THE T CELL PROLIFERATION ASSAY.

The assay, as defined after these studies, was performed on 12 patients (6 MS and 6 neurological controls) and 6 normal controls using human brain microvessels and myelin as antigens (Table R7-1 summarises the characteristics of the patients).

The results of the T cell proliferation study are presented in figure R7-1 for those patients who showed some T cell proliferation against Ag (myelin or vessel)-NCPs over the background and table R7-2 summarises these results. Two lots of AB+ serum were used; the second (used for the last 4 patients) had a much higher background than the first. One MS patient in acute relapse showed significant stimulation against a 55-65 Kd myelin band. Another MS patient in chronic relapse showed stimulation against a 45 Kd vessel band. A patient with progressive MS showed some stimulation against a ~ 68 Kd protein from the brain vessels. The 3 remaining MS patient showed no stimulation or suppression with either myelin or vessels bands tested: these patients were in remission at the time of the testing. Two patients with epilepsy and 1 with anoxic brain damage showed stimulation or suppression with either myelin or vessel band tested: these patients were in remission at the time of the testing. The remaining 3 neurological controls (2 epilepsy and 1 diabetic) did not show any stimulation or suppression with the bands tested.

The results using spotted whole antigen were consistent with the results of the separated NCP in 2 pairs. The remaining pair of patients showed stimulated antigens but not with spotted whole antigen.

The 6 normal controls did not show any significant stimulation against either the vessels or the myelin antigens-NCPs.
<table>
<thead>
<tr>
<th>Numbers in each group</th>
<th>SEX</th>
<th>AGE</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple sclerosis : 6</td>
<td>5 female / 1 male</td>
<td>41 (26-52)</td>
<td>Relapsing-remitting</td>
</tr>
<tr>
<td>MS 1</td>
<td></td>
<td></td>
<td>attack</td>
</tr>
<tr>
<td>MS 2</td>
<td></td>
<td></td>
<td>attack</td>
</tr>
<tr>
<td>MS 3</td>
<td></td>
<td></td>
<td>remission</td>
</tr>
<tr>
<td>MS 4</td>
<td></td>
<td></td>
<td>remission</td>
</tr>
<tr>
<td>MS 5</td>
<td></td>
<td></td>
<td>remission</td>
</tr>
<tr>
<td>MS 6</td>
<td></td>
<td></td>
<td>remission</td>
</tr>
<tr>
<td>Neurological controls</td>
<td>3 female / 3 male</td>
<td>34 (17-57)</td>
<td>non determined</td>
</tr>
<tr>
<td>Epilepsy : 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes : 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain damage : 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal controls : 6</td>
<td>2 female / 4 male</td>
<td>34 (24-60)</td>
<td></td>
</tr>
</tbody>
</table>

**Table R7-1**: Characteristic of the patients and the controls recruited for the T cell proliferation study.
EPILEPSY 2: NO STIMULATION OR SUPPRESSION WITH MYELIN-NCPs OR VESSELS-NCPs.

Figure R7-1a: Stimulation indices obtained from MS patients and their neurological controls with the 11 myelin-NCPs bands and the 17 vessels-NCPs bands. The results are shown by pairs as they were tested (* = stimulation index > 2).
Brain damage: No stimulation or suppression with myelin-NCPs or vessels-NCPs.

Epilepsy 4: No stimulation or suppression with myelin-NCPs or vessels-NCPs.

MS 5: No stimulation or suppression with myelin-NCPs or vessel-NCPs.

**Figure R7-1b:** Stimulation indices obtained from MS patients and their neurological controls with the 11 myelin-NCPs bands and the 17 vessels-NCPs bands. The results are shown by as they were tested (* = stimulation index > 2).
<table>
<thead>
<tr>
<th>MS PATIENTS</th>
<th>CLINI.</th>
<th>MYELIN</th>
<th>VESSELS</th>
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<tr>
<td>MS-1</td>
<td>Remis.</td>
<td>0</td>
<td>16*</td>
</tr>
<tr>
<td>MS-2</td>
<td>Attack</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>MS-3</td>
<td>Remis.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS-4</td>
<td>Attack</td>
<td>4,5,6,9*,10*</td>
<td>8</td>
</tr>
<tr>
<td>MS-5</td>
<td>Remis.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS-6</td>
<td>Remis.</td>
<td>0</td>
<td>0</td>
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<th>NEUROLOGICAL C.</th>
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<tbody>
<tr>
<td>EPILEPSY-1</td>
<td>nd</td>
<td>0</td>
<td>6*,7*,9*</td>
</tr>
<tr>
<td>EPILEPSY-2</td>
<td>nd</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EPILEPSY-3</td>
<td>nd</td>
<td>0</td>
<td>2,[3,5,15,16]*</td>
</tr>
<tr>
<td>EPILEPSY-4</td>
<td>nd</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>DIABETES</td>
<td>nd</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BRAIN DAMAGE</td>
<td>nd</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
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<th>NORMAL CONTROLS</th>
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<tbody>
<tr>
<td>NC-1</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NC-2</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NC-3</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NC-4</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NC-5</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NC-6</td>
<td></td>
<td>0</td>
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</tr>
</tbody>
</table>

Table R7-2: Summary of the T cell proliferation results with the 6 MS patients, 6 neurological controls and the 6 normal controls.
a) Autoantibodies against human brain vessels

The western blots of the brain vessels showed only few sera with autoantibodies: six out 45 MS patients (13%), 8 out of 47 neurological controls (17%) and 1 out of 20 normal controls (5%) had auto-antibodies against vessel bands on western blotting. The diagnoses of the neurological controls with autoantibodies to brain vessels were as follows: prolapsed disc: 1, headache/migraine: 2, vertigo: 1, epilepsy: 4. Most individuals with autoantibodies against human brain vessels show reactivity with more than one band. Figure R8-1 shows the blots of the individuals with autoantibodies against brain vessels (Tracks 1-6: MS patients; tracks 7-14: neurological controls; track 15: normal control). There was no unspecific reactivity recorded against the human brain vessel preparation (track 16 and 17: individual with no antibodies, track 19 and 20: negative controls, 'conjugate' alone) unlike the myelin preparation (see below). Table R8-1 shows the pattern of reactivity by molecular weight.

There is no significant difference between MS and other neurological controls concerning the pattern of reactivity. Nevertheless, 2 MS patients have autoantibodies against human brain vessels against the 64 Kd and 2 other MS patients have autoantibodies against the 97 Kd proteins whereas none of the controls have autoantibodies against these 2 proteins. The other bands with autoantibodies against brain vessels proteins are distributed evenly between MS patients and controls (22, 26, 30, 43, 50, 110, 190, 250, 300 Kd proteins). Although the intensity of the bands are difficult to compare between blots, there is no obvious difference between MS and neurological controls. A rabbit anti-bovine serum was available and reacted against 2 human vessel proteins of around 43kd and 22 kd. The sera of some of the patients with autoantibodies reacted with these 2 proteins (see Table R8-1 and fig R8-1, track 18).
Figure R8-1: Blots of the purified brain vessels incubated with the sera from the MS patients (1-6) [MS], the neurological controls (7-14) [Neurol. Cont.] and the normal control (15) [N] containing autoantibodies to the brain vessels. Strips incubated with one MS patient (16) and one neurological control (17) sera [-] without such autoantibodies are also included. Track 18 [R] shows the reaction with the rabbit anti- brain vessels prepared in the laboratory. Tracks 19 and 20 [-] show the strips incubated with the conjugate alone, (anti-human antibody (track 19) and anti-rabbit antibody (track 20)).
Table R8-1: Molecular weight (kd) of the bands recognised by autoantibodies in the sera of the 6 MS patients (MS1-MS6), the 8 neurological controls (C1-C8), the normal individual (N) (in the same order as figure R8-1) and the rabbit anti-human brain vessels (R).
b) Autoantibodies against myelin:

The reactivity against myelin was more difficult to interpret. Most MS and neurological controls showed reactivity in the 45 to 60 Kd range with different intensity above the background pattern of reactivity (see figure R8-2, tracks 2 to 18). The intensity of the blot with the normal controls was however weak (track 14-17).

On the strip incubated with the 'negative control' (second antibody alone), a band around the 70 kd region was present which therefore was due to non specific staining, perhaps due to endogenous peroxidase activity linked to this band (see figure R8-2, track 17). The band corresponding to the non specific band was present on most of the blots tested incubated with the patients' and controls' sera although its intensity varied greatly between blots. One batch (a blot) did not show this non specific background after development although other bands were highlighted with the sera tested (see figure R2-8, track 1).

Two MS patients and 1 neurological control suffering from migraine showed reactivity with the region corresponding to myelin basic protein and this may be relevant to the disease process (see figure R8-2, track 4,5 and 10).

A group of patients (4 MS and 4 neurological controls) reacted against proteins of medium molecular weight region > 25 kd and < 50 kd but the pattern was variable between patients (see figure R8-2, tracks 6, 11, 12 and 13).

Many patients and controls (80 %) showed reactivity against high molecular weight region (< 150 kd), often as a doublet (see figure R8-2, tracks 4 to 9, tracks 11-13 as well as track 18 corresponding the rabbit anti myelin serum) corresponding to the molecular weight region between 150 and 200 kd and others showed reactivity with even a higher molecular region > 200 kd (5 %) (see figure R8-2, track 1, 2 and 11).
Figure R8-2: Blots of the purified human myelin incubated with the sera from the MS patients. Tracks 1 to 13: MS and neurological controls. Tracks 14 to 16 normal controls. Track 18 shows the strip incubated with the rabbit myelin antiserum and track 17 shows the strip incubated with the conjugate alone (anti human immunoglobulin).
DISCUSSION.

DI: THE CSF STUDIES:

The sensitivity and the specificity of the three CSF techniques (imprint immuno-fixation, immunoblotting and immunoabsorption and silver staining) was analysed in these studies.

All three techniques have been used to detect the production of oligoclonal IgGs in the CSF of MS patients and of other neurologically impaired patients. Immunoabsorption is based on the detection of bands by silver staining. Imprint immuno-fixation and the immunoblotting method rely upon the ability of immunoglobulins to transfer either to agarose gel or nitrocelullose paper respectively and then to be detected by an immunological reaction. The sensitivity of each technique differed markedly with the immunoblotting being the most sensitive. The specificities of the techniques were more difficult to assess. Reproducibility, to some extent, correlates to specificity. The silver staining detection of oligoclonal IgG was a reliable method when the concentration of immunoglobulin was high in the CSF. The band pattern was clear but some additional band(s) sometimes appeared with variation in the value of the electric current. These bands were artefacts and could be seen across the whole gel and between tracks. Normally, the method was reproducible if the same CSF was tested repeatedly.

Using another step for each of these CSF techniques, one can also assess the prevalence of viral specific immunoglobulins in a CSF. The sensitivity of each technique was the same as when used to detect the oligoclonal response. However, in the detection of viral specific immunoglobulins none of the methods were totally specific.

Oligoclonal IgGs produced in the CSF of MS patients are strikingly cathodic. Heterogeneity of charges among immunoglobulins has been known for quite a long time. It has been shown that charge properties of IgE antibodies inversely correlate, to some extend, with the charge of the corresponding antigens [Underdown B.J. et al, 1970]. It has also been observed that charges of IgGs correlate in the same inverse way to the charge of the corresponding antigens [Levy D.E. et al, 1981]. It is therefore tempting to suggest that the antigens corresponding to the oligoclonal response in MS are mainly negatively charged.

Concerning the immunoabsorption technique, it was demonstrated that the relative cathodic oligoclonal immunoglobulin
G's appeared to be sensitive to changes in phosphate ion concentration and in pH. Therefore, it may be that some of the absorptions noted in the original series of experiments done by Dr K. Goswami were due to non specific immune reactions. It is therefore possible that depending on the particular viral preparation used, certain CSFs may have had their biological properties e.g their charges modified and were thus more prone to precipitate. However, this cannot explain all the findings reported and published in the article in Nature [Goswami K.K. et al, 1987]. For example one particular CSF had a very high level of antibodies to the viral protein HN (a protein which is known to be very specific for SV5 and not to cross react with the HN protein of the related virus PF2). This CSF was not available for further study but it may be that a proportion of CSFs - much smaller than previously thought - may still possess a significant portion of their oligoclonal IgGs specific for SV5 [Russell W.C et al, 1989]. Only, an extensive study on a very large number of CSFs will answer this question. Brankin and colleagues [Brankin B. et al, 1989] tested SV5 antibodies by the ELISA technique in the CSF of MS patients. They found that 40 % of the patients tested (total number of patients tested: n=13) had specific antibodies to SV5. However they found that the elevation of SV5 antibodies correlated to some extent to the elevation of measles antibodies in these CSFs and concluded that the response specific to SV5 was part of the non specific anti viral response found in the CSFs of MS patients.

The specificity of the other 2 techniques (immunoblotting and imprint immuno-fixation) were also questionable. The immunoblotting technique gave results difficult to interpret when a defined antigenic system and corresponding monoclonal antibodies was used. Moreover, the results obtained with the human CSFs against different viral antigens were also difficult to interpret in some cases. In practice positive controls (usually mouse monoclonal antibodies) or specific controls are included in an analytical system when testing human CSFs but it is claimed that mixing conjugate antibodies of different allotypes may disturb the result of the test [B. McLean, personal communication]. Therefore no mouse monoclonal antibody specific to a particular virus is included when testing human antibodies specific to this virus in CSFs. In conclusion the immunoblotting technique is not a recommended method of detection of antigen specific IgG in the CSF. Nevertheless, as a diagnostic tool
for the detection of oligoclonal IgG in the CSF of neurological patients, this technique appears to be the most sensitive and as specific as the other two. Only a proper large scale clinical study would detect the proportion of false positive and negative results experienced with this technique. This will be difficult to achieve as there is no gold standard method in CSF characterisation.

The results obtained by Dr Vandvick and Professor Norrby with the imprint immuno-fixation method appeared quite specific and the correct controls were included in each experiment [Vandvik B. et al, 1989]. This discrepancy of specificity between the St Andrews' group with this technique and the Scandinavian group may be due to the lack of experience of the St Andrews' group with this technique. One of the factors which influenced the specificity of the technique was the time of overlay between the agarose gel (where the CSF have been electrofocused) and the antigen containing gel. The other important point is that the technique is not very sensitive and the CSF must be concentrated before the electrophoresis. Some have suggested that critical IgG could be lost during concentration of the CSF e.g. absorbing to a dialysis sac. Others have shown that the loss of protein during concentration is not restricted to particular proteins and thus there is no reason to believe that critical IgGs are preferentially lost during dialysis [Walsh M.J. et al, 1983][Kaplan A. et al, 1966].

However, the preparation of the antigen in hot agarose may have been a source of loss of specificity in their otherwise well controlled experiment. In order to mix the antigen within the agarose, the agarose has to be molten at 56 °C, and this temperature may potentially have altered some epitopes. It is possible that epitopes which are critical in MS patients can be destroyed by this process and this is especially relevant for the membrane glycoprotein HN of the SV5 virus as it appears that most of the epitopes on this glycoprotein are conformational [Goswami K.K. et al, 1987]. On the contrary, the epitopes of the internal proteins of the virus e.g NP and P are less sensitive to external treatment. This may explain why some cross reactivity was observed between PF-2 and SV5 with some MS CSFs as internal proteins of these 2 viruses cross react [Randall R.E. et al, 1988]. However, it is possible that the critical response to the HN glycoprotein was not detected because of thermal inactivation of virus antigens. From the previous work, HN has been
described as the very protein which is recognised by the oligoclonal response in some MS patients [Goswami K.K. et al, 1987].

In conclusion, none of the 3 techniques was specific when examining antigen-specific IgGs in the CSF. The most sensitive method of detection of oligoclonal IgG is the immunoblotting method. New methods should be designed to study antigen specific immunoglobulins in the CSF.

D2: CULTURE OF ENDOTHELIAL CELLS

Although culture of rodent or bovine brain endothelial cells seems to be well established by some research groups [Bowman P.D. et al, 1983][Audus K.L. et al, 1986][Wilcox C.E. et al, 1989], there are few reports of culture of human brain endothelial cell lines [Gerhart D.Z. et al, 1988][Vinters H.V.et al, 1987] and these have so far been limited to the description of the culture conditions.

The aim of this project was to grow human brain endothelial cells in culture principally because a source of human brain endothelial antigens would facilitate the use of clinical material. The brain endothelial cells could have been used as a source of antigens for immunisation in order to produce polyclonal and monoclonal human brain-specific antibodies. The cell line would have been also a good source of antigens for a monoclonal screening procedure. Moreover, the analysis of expression of adhesion molecules on human brain endothelia may have given some information relevant to the pathogenesis of multiple sclerosis (see the introduction section: the brain vessel in MS).

The culture of human brain endothelial cells appears very difficult to achieve from the work reported in this thesis. This is partly due to the lack of human fresh brain tissue for such experiments. Gerhartz and colleagues also used human brain tissue removed at surgery as a source of tissue for growing brain endothelial cells in culture [Gerhartz D.Z. et al, 1988]. However, Vinters and colleagues reported that they were able to grow endothelial cells in culture from brain tissue removed from post mortem material [Vinters H.V. et al, 1987]. In this present work, only culture of endothelial cells from surgical material was tried because it was felt that the chance of obtaining viable material was greater from this source. There are about 200 temporal lobectomies
performed a year in the U.K and 2 were done in Dundee during the
time of this project on brain endothelial cell culture (it appears that
the indication for temporal lobectomy varies slightly between
surgical departments with some neurosurgeons more interventionist
than others).

The first attempt of growing human brain endothelial cells in
culture was unsuccessful. It was therefore decided to use brains from
animals and as bovine brains had been used successfully by others
for the preparation of the brain vessels [Audus K.L. et al, 1987] and
some preliminary experiments were attempted using material
obtained from the local slaughterhouse. However, at the time of the
project bovine spongiform encephalopathy (BSE) was an almost
everyday news headline and it was felt that it would be more prudent
to use another source of animal brain. Since rat brains were
available in the department from the neurochemistry group these
were used as a source of rodent microvessels.

The principle of growing endothelial cells in culture is first to
purify the brain vessels, then to partially disrupt the overall
structure of the vessels with collagenase and then to culture these
vessels in tissue culture flasks in order to let endothelial cells grow
from the purified vessels. The main problem encountered was that
the vessels after purification did not stick to the tissue culture flasks
and were seen floating after 2 days in culture. The reasons for this
inability to stick to the plates are not known but different
possibilities were discussed and some were tested. The first
possibility is that purified vessels need a long time (more than 2
days) in order to stick to tissue culture plates. It is known that vessels
purified from the omentum take a long time to seed on tissue culture
plates (J Pearson, C.R.C Northwick Park, personal communication).
However, it is is unlikely in the case of purified brain vessels as after
more than 2 days in culture the purified vessels appeared completely
disrupted and not viable. It is more likely that something which
could have promoted seeding of the vessels was missing from the
experiment. Some researchers have suggested that vessels should be
incubated in the tissue culture flask in a minimal volume to promote
adhesion (S. Kumar, Manchester, personal communication). Only
once in the cited references was the problem of adhesion of purified
vessels to culture dishes discussed by the authors. When Forrester
and colleagues tried to grow endothelial cells from purified vessels
of bovine retina on fibronectin-coated coverslips , they observed that
the vessels had a tendency to detach from the coverslips. They then used a highly viscous substance, sodium hyaluronate (Pharmacia) to cover the purified vessels on day 0 of culture. The sodium hyaluronate slowly dissolved in the culture medium and was only removed when the medium was changed. In this way, the adherence of the vessels was achieved on the coverslips [Roberts J.M. et al, 1990][Hughes C.C.W. et al, 1986].

Another factor which is thought to promote seeding is the preliminary treatment of the purified vessels by collagenase. Collagenase treatment disrupts the extracellular matrix of the vessels and is likely to disclose reactive groups which may promote seeding. Other factors which also may help the seeding are the attachment factors which may be used to coat the tissue culture flask: they all mimic an extracellular matrix [Russo R.G., 1986]. Only gelatin was used as an attachment factor in our experiments. Other attachment factors for the coating of the culture petri dishes may be more suitable. Some research groups have used commercial extracellular matrices like 'basement membrane Matrigel' (collaborative research) [Gerhart D.Z, 1988], extracellular Matrix -ECM-coated culture dishes (Eldan technologies) [Shivers R.R., 1988] or Falcon primaria culture plates [Lathey J.L. et al, 1990]. Other attachment factors, alone or in combination have been used e.g collagen [Hughes C.C.W. et al, 1986][Bowman P.D et al, 1980], collagen + fibronectin [Audus K.L. et al, 1987] or fibronectin alone [Bowman P.D., 1983].

Forrester and colleagues found that fibronectin and laminin provided excellent substrates for bovine retina endothelial cells in culture but in contrast plasmin and collagen were poor substrates [Roberts J.M. et al, 1990][McIntosh L.C. et al, 1988]. Others have prepared their own culture dishes plates coated with extracellular matrix by culturing cells producing extracellular matrix on the culture dishes and then disposing off the cells to obtain an extracellular matrix coated plate [Meresse S. et al, 1989] [Gospodarowicz D., 1984].

It is also possible that different media could promote the adhesion of vessels e.g the different ion content could modify the activities of certain proteases involved in the adhesion process or in the disruption of the extracellular matrix. In the same way, vessels may need to be purified in magnesium or calcium free medium - ions known to influence the activity of certain proteases. Different media have been utilised to grow brain endothelial cells e.g M 199, F 12, Ham's media.
Other factors may influence the success of a culture of brain endothelial cell especially when the vessels have adhered and cells have begun to grow from them. Pericytes, which are not endothelial cells but are thought to be of smooth muscle lineage, may contaminate the culture. Some research groups have advocated the use of plasma derived serum (PDS) in place of normal serum [Audus K.L. et al, 1986]. It seems that PDS promotes the growth of endothelial cells and inhibits the growth of contaminating pericytes as PDS is characterised by the absence of some soluble factors [Vogel A. et al, 1978]. Others have used an anti-Thy1.1 with complement to kill contaminant cells in order to grow rat brain endothelial cells [Risau W. et al, 1990].

The use of other types of sera have been advocated especially human serum for some endothelial cell culture and the proportion of serum in the culture may be critical e.g it appears that dermal microvascular endothelial cells require very high (40-50%) serum concentrations [Pearson J., 1990].

It has been demonstrated that tumors produce endothelial growth factors as they need blood supply when they increase in size [Folkman J. et al, 1980] and some research workers have used tumor-conditioned media to promote the long term growth of endothelial cells in vitro [Folkman J. et al, 1979]. Again there is no consensus on which tumors cells are good producers of endothelial cell growth factors and exactly what type of endothelial cell growth factor is produced. Different cells have been used e.g murine melanoma cells (K1735-M2) [Gerhart D.Z. et al, 1988] or sarcoma 180 cell line [Folkman J. et al, 1979].

In the same way, it is thought that glial cells and/or glial factors secreted by glial cells promote the brain specific properties of these endothelial cells [Goldstein G.W., 1988]. Thus it was decided that brain endothelial cells should be grown in glial-conditioned medium and to achieve this we decided to grow glial cells in culture from neural biopsies. This was done following the method described by Bama and colleagues which is based on the principle that adherent cells grown from primary culture of a brain biopsy of an astrocytoma are likely to be glial cells [Barna B.P. et al, 1985]. However, the cells obtained in our study did not express the specific glial marker -glial associated fibrillary protein. It has been well reported that glial cells can lose their glial-specific marker.
(GFAP) when they are cultured and that the prevalence of GFAP-expressing cells can vary with cell density and differentiation [Frame M.C. et al, 1984]. It is thus possible that the cell lines grown in culture were in fact glial cells which did not express GFAP in culture because of their differentiation or because they had lost this marker in very early passages. Another explanation is that the method of purification followed [Barna B.P. et al, 1985] was not as stringent as the other published method of glial cell purification [e.g Selmaj K.W. et al, 1990] and that the cells obtained were not glial in origin but contaminant cells e.g fibroblastic in origin. It is however extremely difficult to be sure of the fibroblastic origin of cells by immunohistochemistry as there is no specific marker for fibroblast cells. Indeed the intermediate filaments expressed by fibroblast e.g vimentin are also expressed by other cells. Cheng-Mayer and colleagues used a monoclonal antibody thought to be specific for fibroblastic cells and which recognised procollagen III [Cheng-Mayer C. et al, 1987]. However this monoclonal antibody was not available in the U.K.

Therefore, as the home-grown 'glial cell lines' were not satisfactory, we assessed glial cell lines already characterised and available from colleagues. U 138, a glioma cell line, was given by Professor Weiss and CM-C-CM, also a glial cell line, was purchased from the European cell culture collection (Porton Down, Salisbury). They were tested for the expression of the marker GFAP - less than 10 % of the cells of both cell line were positive for the marker. This is again in accord with previous reports which described the loss of the glial specific marker by glial cell grown in culture [Frame M.C. et al, 1984].

In conclusion, growing human brain endothelial cells in culture may be possible when all conditions are met (medium, attachment factor, optimum digestion of purified vessels, endothelial growth factors and glial growth factors). However both brain endothelial cells and glial cells (source of glial factors) may loose their specific markers in culture and therefore proof of the origin of a cell line may be difficult. In the same way, very little is known about the factors influencing the loss or gain of endothelial and glial cell markers in culture although some factors have been studied in relation to glial cells [Frame M.C. et al, 1984].
D3: THE 'NITROCELLULOSE T CELL ASSAY'

The idea of separating proteins from a crude source of antigens on a SDS PAGE gel in order to facilitate the study of possible autoantigens is not new as it has been used in conjunction with the 'Western blotting' technique to detect autoantibodies in a number of autoimmune conditions [Lynch D.M. et al, 1986][De Keyser F. et al, 1989] or in paraneoplastic syndrome which are thought to be autoimmune in origin [Babikian V.L. et al, 1985]. However, study of T cell reactivity tested by a T cell proliferation assay was based on the use of soluble antigen. The 'NTP T cell assay' has been described recently and may allow researchers to study autoimmune T cell reactivity using antigens separated by molecular weight by SDS-PAGE gel electrophoresis. The principle of the technique was first described by Laurent and colleagues who demonstrated proliferation of T cells after stimulation by phytohemagglutinin (PHA) separated after SDS-PAGE electrophoresis and electroblotting transfer to nitrocellulose membranes [Laurent B. et al, 1985]. Lamb J., Young D. and colleagues followed this idea by using different antigens particularly proteins from influenza virus, M. tuberculosis and allergens [Young et al, 1986][Lamb J.R. et al, 1987, 1988]. They used the same principle as described by Laurent and colleagues and called the technique a 'solid-phase antigen T cell' assay. In their assay, the antigens separated on SDS-PAGE electrophoresis were blotted on nitrocellulose paper (Ntcp), the 'band' corresponding to the antigen of interest was cut out and the piece of Ntcp with the antigen directly incubated in a well of a microtiter plate in order to perform a classical T cell proliferation assay. Abou-Zeid and colleagues described an improvement of the technique. Instead of incubating the piece of Ntcp containing the antigen of interest in the well, they first dissolved the piece of Ntcp in DMSO and then reprecipitated it into a suspension of antigen-bearing particles by adding carbonate buffer [Abou-Zeid C. et al, 1987]. Serendipity played an important part in the discovery of the buffer: the buffer chosen for reprecipitation was that used in ELISA buffer assay protocols (G. Rook, personal communication). The antigen-bearing particles appeared to be taken up readily by antigen-presenting cells and induce a significant proliferation with very small amounts of antigen. With mycobacterium leprae as antigen, Abou-Zeid and colleagues found
that 0.1 μg of antigen-bearing NCP per well gave an optimal stimulation in a T cell proliferation assay whereas the same soluble antigen gave an optimum stimulation at 2 μg per well.

The 'NTP T cell' assay had to be assessed and standardised in our laboratory in order to be able to use this assay confidently for a clinical study.

a/ STANDARDISATION OF THE 'NTP T CELL' ASSAY.

When starting to use the 'NTP T cell assay' there are 2 potential problems - contamination and non stimulation by the antigen-bearing particles. Possible contamination of the T cell assay by the antigen (antigen-bearing NCPs) is indeed possible as it is processed in a non sterile fashion through a cycle of gel electrophoresis and electroblotting and prepared with a non sterile NTP. However, contamination with antigen-bearing NCPs has never been encountered during the work related to this thesis. Contamination is often detected in a T cell assay when the counts of a test-sample in triplicate are distorted for example with one well giving very high background whereas the other wells containing the same sample give low or intermediate counts. When the technique described in Material and Methods is followed, the antigen preparation goes through a DMSO step which it is thought to sterilise the antigen preparation. All the next stages of the technique are performed in a sterile fashion.

The second problem was experienced during the course of this thesis and is concerned with the non stimulation of potential antigen specific T lymphocytes when these T cells are apparently incubated with the appropriate antigen-bearing NCPs. Many factors which could influence this non stimulation were assessed (see the Results section) but only one was found to be critical and this was the size of the antigen-bearing particles. The optimum size can be assessed very easily after resuspension of the antigen-NCPs by carbonate buffer. The sedimentation of the particles can be seen and the time of sedimentation appears to correlate inversely with the capacity of inducing stimulation to antigen specific T cells. Optimum results were achieved when the reprecipitation was carried out under control conditions (when the carbonate buffer was delivered at a constant speed of 0.8 ml/min using a peristaltic pump with NTP dissolved on DMSO stirred on an electric magnetic stirrer, at constant and reproducible speed). It is thought that the small size of
the nitrocellulose particles obtained allowed them to be properly phagocytosed, processed and then presented by the antigen-presenting cells in the T cell proliferation assay.

One factor which was also thought to be critical was the possible contamination of the Ag-NCPs with DMSO - inducing inhibition of stimulation in a standard T cell assay. However washings performed and described in the Material and Methods section appeared to be adequate. They were also performed at 4 °C because it was thought that, as this washing procedure was lengthy (4 washes of 10 minutes each), it would help preserve the optimal antigenicity of the Ag-bearing NCPs.

To achieve the standardisation of the 'NTP T cell assay', the 'spotting technique was used as it is a simple technique which allows the antigen, spotted directly on Ntcp, to be processed readily into Ag-NCPs without the electroblotting step. This reduced the time and efforts involved in the preparation of different Ag-bearing NCPs. On the other hand it cannot provide data on proteins separated by molecular weight and thus, in itself, is a procedure with little advantage over a classical T cell proliferation assay with soluble antigen. However, the amount of antigen needed to induce a proliferative response as compared to a classical T cell proliferation assay (see results section) is less and, thus, an economy of antigen can be achieved with the same end result when one is testing a 'precious' antigen in a T cell proliferation assay.

On the subject of optimal concentration of the antigen, it is likely that proteins are to some extent lost during the preparation of the NCPs (for example during the washing) and therefore the real concentration of protein into each well may be much lower that calculated. Radioactive labelled proteins processed into Ag -NCPs could give data on this question; these types of experiments were not done in this study. A series of experiments was performed in order to analyse the effect of washing on antigen blotted or spotted on Ntcp and interesting results were obtained which have implications not only for the 'NTP T cell assay" presently discussed but also for other techniques involving transfer of proteins to Ntcp e.g 'western blotting' or immunodot-blot. From the results presented, it is apparent that proteins spotted on nitrocellulose paper do not behave like proteins electroblotted - presumably the latter are much more firmly bound to the nitrocellulose paper. This is important when using a washing buffer which contains detergent which may wash
away the proteins spotted on nitrocellulose paper and thus this factor has to be taken into consideration for any assay which uses spotted protein on nitrocellulose paper e.g immunodotblot.

After standardisation of the 'NTP T cell' assay, it was noticed that the antigen concentration needed to induce a proliferation and the optimum time of incubation for this optimum proliferation differed between a classic T cell assay and a 'NTP T cell assay. Comparing with a classic T cell assay, the optimum concentration of antigen is much less in a 'NTP T cell' assay. However the optimum time is longer, about 5-7 days, compared to 4-6 days for a classic T cell proliferation assay. Data presented in the Results section also show that the optimum stimulation index is lower with antigen-bearing nitrocellulose particles than with soluble antigen. The explanation for these phenomena is not known. The lower antigen concentration needed in a 'NTP T cell' assay has been reported by all the groups who have used the method [Abou-Zeid C. et al, 1987][Filley E. et al, 1989][Lee S.P. et al, 1989]. The particulate structure of the antigen (as antigen-bearing nitrocellulose particles) is likely to be critical and the antigen coated on a particulate matrix may help the antigen presenting cell (APC) to phagocytose the antigen more readily.

As already mentioned above, the optimum time of stimulation appears longer with antigen coated particles than with soluble antigen. This observation has been made by other groups. Moreover, those who have used T cell clones in a 'NTP T cell' assay have also reported the same trend of a longer incubation time when compared to T cell clones in culture with soluble antigen. The optimum time concentration for the former is about 4 days whereas it is 3 days with the latter (Anne Rees, Hammersmith Hospital, personal communication). The fact that overall incubation time for T cell clones is shorter than for peripheral blood mononuclear cells may be explained by the fact that the effector T cells are monoclonal and therefore, proportionally, the frequency of antigen-specific T cells in the well is much higher than in a polyclonal system (e.g with peripheral blood mononuclear cells). However the fact that it takes more time to observe an optimum proliferation in a 'NTP T cell' assay compared to a classic T cell assay is difficult to explain. Taking into account the lower optimum concentration in a 'NTP T cell' assay compared to the classic assay and the lower stimulation index with the 'NTP T cell' assay, it appears that the antigen is more
efficiently captured and phagocytosed by the antigen presenting cells (less antigen needed) but it takes longer for the antigen to be processed (longer incubation time) and the proliferation is less intense (lower stimulation index), the latter suggesting that there is less antigen presented at the surface of the APC or that most of the antigen is presented by the wrong MHC class e.g. class I MHC molecules instead of class II MHC molecules for a T cell proliferation assay. The lower stimulation index could also be explained by a negative effect of the nitrocellulose particles on the proliferation independent of the antigen specific effect. This is unlikely as it was demonstrated that nitrocellulose particles incubated with soluble antigen do not induce a negative effect on the T cell proliferation due to the soluble antigen (see Results section). In the same way, the possibility of stimulation by a superantigen which could be present on nitrocellulose is very unlikely as negative control nitrocellulose particles do not induce proliferation in the standardised system (superantigen stimulation is not mediated via the specific recognition of the antigen by the T cell receptor on a HLA restricted manner but by reaction of the antigen directly with the Vβ segment of the T cell receptor [Acha-Orbea H. et al, 1991]).

In conclusion, the 'NTP T cell' assay was standardised and a number of points about the techniques have been shown to be critical. The 'NTP T cell' assay is reproducible with characterised antigens and is a useful tool for studying T cell response to proteins separated by molecular weight. However, it is recommended that one should start with the spotting technique especially when standardisation of a previously unknown system is necessary. It is also recommended that an assessment, in a mock experiment with MOCK-NCPs, of the optimum precipitation of the NCPs with carbonate buffer while changing the volume of DMSO: the optimum quantity of DMSO depends on the volume of NCPs processed (see Results section).

b/THE CLINICAL STUDY USING THE T CELL ASSAY:

Autoreactive T cells are thought to be the main components of the proposed autoimmune reaction in multiple sclerosis (MS). Considerable attention has been focussed on myelin basic protein (MBP) since experimental allergic encephalomyelitis (EAE) can be induced by MBP. Surprisingly little work has been done to study the T cell response with other myelin proteins. As discussed in the
Introduction section, the blood brain barrier may also be a target for a T cell response. The next part of the thesis therefore tested the possible T cell reactivity of MS patients against different antigens from both the myelin and the brain vessels using the 'NTP T cell' assay. It was decided to use the T cell proliferation assay which is mainly an indicator of the T helper cell response. Most of the T helper lymphocytes are CD4 positive cells and thus are restricted by MHC class II molecules (the CD4 antigen on the T cell making contact with the class II MHC molecule on the antigen presenting cell). The genetic association described with the MHC system in MS patients has been strongly linked to the MHC class II locus (especially DR 2 and DQw1). If T lymphocytes are involved in the MS process, class II restricted T cells are therefore likely to be the main culprits and it is thus more appropriate to utilise a T cell proliferation assay which analyses the T helper cell response in MS studies. Moreover, if class II restricted cytotoxic T cells (instead of T helper lymphocytes) are the main culprits in the MS process, it is likely that T helper lymphocytes related to these cytotoxic responses will be detectable; the cytotoxic T cell response is mainly T helper cell dependent as are the B cell response [Cantor H. et al, 1976]. A T helper cell first recognises the antigen and will produce soluble factors which will help to generate a cytotoxic response directed against this same antigen [Balkwill F.R. et al, 1989][Hamblin A. et al, 1988].

The source and the purity of the antigen incubated in the assay is also a limitation to many T cell proliferation assay. It is extremely difficult to completely solubilise proteins from purified brain vessels. It was also thought that if satisfactory solubilisation is achieved, it will be extremely difficult to separate different molecules from one another unless long purification procedures were used. Thus the only way to test the vessel molecules in a T cell assay would be to incubate the total solubilised proteins in each well. Two main limitations were discussed at the time in relation to this possibility. First, the concentration of each molecule would be difficult if not impossible to predict. T cell proliferation is dependent on the antigen concentration as the curve of stimulation is a bell shape curve. In a classic T cell assay normally 1 to 100 μg. of pure protein induce an optimum proliferation response. If one starts with a very heterogeneous preparation of antigens and if only one of the antigens is recognised by T cells as part of an autoimmune
response, it is very difficult to find this optimum concentration or at least a concentration of antigen which will induce a proliferation. Secondly, if one uses the whole solubilised brain vessels as antigens in a T cell proliferation it is likely that MHC class I and perhaps class II molecules will be present in the preparation as both classes of MHC molecules may be expressed on the brain endothelial cells, the class II molecules being expressed only if the brain endothelial cells are treated with certain cytokines e.g. γ-INF and IL2 (see BBB section in the introduction). It is thus possible that these MHC molecules will induce an alloreactivity in a T cell proliferation assay where the T cells are from a MS patient (or from a neurological control) who is likely to have a different HLA status from the postmortem patient whose brain vessels act as antigen in the T cell proliferation assay.

It was therefore decided to adapt the recently described 'nitrocellulose T cell assay' for the study of autoantigens in autoimmune diseases. This technique had never been used in MS studies before or in any studies of autoimmune diseases. The 'NTP T cell' assay may allow the HLA molecules bands to be eliminated after their localisation either by immunological method (for example by Western blotting technique' with an anti-HLA class I and/or class II molecules) or in relation to their molecular weights (MHC class I molecules have a molecular weight of around 43 kd whereas the both class II chains have a molecular weight of 28 kd). On the other hand, standardisation of the concentration for each antigen band is difficult to achieve even if the 'NTP T cell' assay may allow more flexibility than a classic T cell assay with a heterogenous antigen preparation e.g myelin or brain vessels. When proteins of brain vessels or myelin are stained with naphthalene black after electrotransfer, the quantity of each proteins is very heterogeneous. However, it may be possible to select a band of interest and load the appropriate antigen in amounts relating in function of this band of interest e.g if the protein is not very well represented on the original gel, it may be possible to "overload" the minigel and thus concentrate to some extent on the protein of interest whereas if the quantity of the protein of interest is high, it is possible to load less of the original concentration of the whole preparation. This possibility of this modulation was not explored in the thesis.

The results obtained with the MS patients and the neurological controls, using only peripheral blood lymphocytes, were not
definitive although some patients showed minor T cell proliferation against a number of components of the myelin or of the brain vessels. This reactivity tended to correlate with activity of the disease in the multiple sclerosis patients as only the 2 patients who were suffering from an attack, at the time of the assay, showed proliferations against myelin or vessels components. The other 4 patients who were in remission at the time of the assay did not show any proliferation. Interestingly, among the neurological controls, some patients showed proliferation against components of the brain vessels and these patients suffered from epilepsy (although it is difficult to draw any statistical conclusions as the number of the neurological controls tested is low (n=6)). None of the normal controls showed any reactivity to either myelin- or vessel- NCP. The same trend was observed with the Western blotting study as 14 patients (15%) showed reactivity towards some of the proteins of the the brain vessels whereas only one normal control (5%) did. The reactivities seen in both the T cell assay and the western blotting may be consequences of the disease as it may be that an immune response is triggered when there is release of brain components during the pathological process e.g in multiple sclerosis or in epilepsy. The results using spotted whole antigen were consistent with the results of the separated NCP in 2 pairs. The remaining pairs of patients showed stimulated antigens but not with spotted whole antigen. This is to be expected as whole spotted antigen has been shown to produce less stimulation than separated antigens in some cases e.g with the mycobacterium tuberculosis system [Lee S.P et al, 1989].

Overall, the stimulation indexes observed were low. It is difficult to know if this was due to the overall lack of response to the antigens tested by the patients or to the technique used - the 'NTP T cell' assay. It has already been discussed that overall, stimulation indices obtained with the 'NTP T cell' assay are lower than those obtained with soluble antigens. In addition, in the clinical study described, only one concentration of antigens for each band was tested and therefore it is possible that for the various myelin and brain vessel proteins, the optimum concentrations were different even though the corresponding nitrocellulose particles prepared were done following standardisation of the assay. The standardisation of the 'NTP T cell' assay was done with known protein systems, mainly M. tuberculosis, tetanus toxin and
adenovirus type 2 proteins but brain vessel and myelin proteins may not 'behave' in the same way at the defined optimum concentrations.

However, it is also possible that there was no 'high responders' to myelin or brain vessels tested in the population tested. Johnson and colleagues tested the reactivity of peripheral blood mononuclear cells from MS patients and neurological controls against soluble purified MBP, PLP and MAG. Only about 15% of the patients were high responders (mainly against MAG and MBP) whereas the rest did not produce stimulation indexes above 2 with the proteins tested [Johnson D. et al, 1986]. Consequently, the frequency of autoreactive T lymphocytes in the peripheral blood compartment may be too low in most of the MS patients and thus amplification techniques e.g T cell cloning may be useful to expand the population of autoreactive T lymphocytes in the compartment tested.

One important point on the clinical T cell assay study is that the human serum used in the T cell assay gave a high background. The background interferes with the proliferation in terms of stimulation index. If an antigen is incubated with antigen-specific T lymphocytes in a medium which gives low background, the stimulation index is much higher than when this antigen is incubated in a medium which gives high background (see Results section). Therefore some significant antigen-specific proliferation may have been missed in the clinical T cell study (no 'low background' serum was available at the time of the study - twenty mls of low background serum was obtained for the standardisation of the 'NTP T cell' assay (see Results section, figure R6-2a, page 199) but none was left for the clinical study). Retrospectively it would have been proper to use autologous sera for the clinical T cell assay because autologous serum usually induces low background count (Dr Luis Carlo-Branco, C.D.U, St Georges Hospital, personal communication). However at the time of the study it was thought that a unique batch of AB serum would keep the conditions of culture standardised between the different patients tested.

The other possibility to explain the low stimulation index is that multiple sclerosis is not an autoimmune disease and that the T lymphocytes in the lesions do not react with autoantigens. Some may be specific for viral (or bacterial) antigens and there is very little or even no reaction against brain autoantigens (see introduction Viruses and MS section). This appears to be the case in some viral
demyelinating diseases where most of the myelin damage appears to be mediated by soluble factors as a bystander effect.

Nevertheless, the author still considers the 'NTP T cell' assay as a potentially useful assay for the study of autoimmune disease and particularly multiple sclerosis. Similar studies using peripheral blood lymphocytes, 'low background' serum and fewer antigens (increasing the possibility of testing different doses for each antigen) have to be performed with more multiple sclerosis patients. In addition, this technique could be very useful for testing the specificity of T cell clones derived from the CSF or even from the brain of post mortem patients. The peripheral blood is indeed only a 'window' on the lymphoid system and blood lymphocytes are released from and migrate into disease tissue [Westermann J. et al, 1990]. Other than the peripheral blood the different sources of T cells available for studying the T cell response in MS are the CSF, the target organ itself e.g the MS lesions and possibly the lymph nodes related to the brain. It is likely that possible autoreactive T cells will be concentrated at the site of the lesions and it is thought that the CSF may reflect more closely the T cell population of the brain than the peripheral blood [Calder V. et al, 1989][Hafler D.A et al, 1989]. However, there is still much debate about the compartment in which the autoreactive T cells may be found - blood versus brain [Calder V. et al, 1989][Hafler D.A. et al, 1989].

There are two ways of raising T cell clones/lines [Fathman G. et al, 1989]. Commonly, this is done in an antigen specific fashion by raising T cell clones with IL2 in the presence of the antigen thus promoting only the T cells specific for the antigen of choice e.g see [Richert J.R. et al, 1986][Sredni B., 1982]. However, the other technique which is less commonly used is to raise T cell clones in the absence of antigen, using only IL2 and a mitogen [Moretta A. et al, 1983 a and b] , therefore, theoretically only inducing the long-term culture of activated T lymphocytes present in the sample collected for the preparation of these T cell clones [Hafler D.A. et al, 1987, b]. This technique has been used to test T cell clones raised from the CSF of MS patients [Fleisher B. et al, 1984][Hafler D.A. et al, 1985] [Hafler D.A. et al, 1987] and one report described the successful preparation of T cell clones from the lymphocytes collected from post mortem plaques from 2 MS patients. However, only MBP, PLP and myelin associated glycoprotein (MAG) antigens were tested in a classic T cell proliferation assay and no proliferation of these T cell
clones was measured with these antigens [Hafler D.A. et al, 1987].
The 'NTP T cell' assay could provide a mean of increasing the panel
of antigens which can be tested provided that there are enough
cloned T cells and antigen presenting cells to performed the test.

D : THE CLINICAL STUDY USING THE WESTERN
BLOTTING TECHNIQUE.

There are many advantages of the Western blotting procedure
(SDS PAGE followed by Western blotting) [Towbin H. et al, 1984].
The preparation used as a source of antigen can be crude e.g a piece
of tissue or cells grown in tissue culture or partially purified
components from a tissue. Secondly, the 'Western blotting' technique
is to some extend analytical as the proteins are separated by
molecular weight but it may also be used as a screening procedure as
many sera can be tested in parallel against a unique antigen.
Sensitivity of the technique cannot be considered alone but only
when also considering specificity. These 2 factors depend on each
system studied principally on the definition of the negative controls.
When the question of negative and positive control has been
overcome, the sensitivity of the technique is satisfactory and depends
on the visualisation system used e.g peroxidase or radioactive probe.

The main disadvantages of the method is the preparation of the
samples which involves denaturation of the proteins before the SDS-
PAGE gel electrophoresis: the samples are disrupted in SDS with or
without mercaptoethanol (reduced or unreduced mixture
respectively). This procedure can destroyed critical epitopes on the
antigen of interest especially if these epitopes are conformational
[Horsfall A.C. et al, 1991]. In the same way, the conditions of
transfer of the antigens from the SDS PAGE gel onto nitrocellulose
may also alter certain epitopes for example due to heat or to the
buffer used during the transfer procedure [Towbin H. et al, 1984].
Also, it has been shown, in some system, that blocking solutions
influence the antigenicity of the proteins transferred on

On the other hand, the antigen preparation used in this study
was considered to be adequate as the purified vessels expressed von
Willebrand factor and \( \gamma \) glutamyl transpeptidase. Moreover, the
vessels were prepared from a post-mortem brain less than 36 hours
after death and Choi et al showed no difference in the protein pattern and functional integrity between rabbit brain capillaries isolated either at 0 or 42 hours post mortem [Choi T.B. et al, 1986].

When considering possible autoantigens recognised by autoantibodies on Western blotting, one has also to ask if these autoantibodies are relevant for the disease process. Indeed, some have suggested that some autoantibodies are 'physiological' and are part of the normal equilibrium of the immunological network, this network being part of a bigger system which has been called *milieu interieur*. For example, it has been suggested that some 'autoantibodies may have a role in disposing of the product of metabolism and catabolism [Grabar P., 1983]. This may be particularly relevant to some autoantibodies detected by Western blotting after disruption of the antigen. Some may not be 'harmful autoantibodies' but autoantibodies involved in the clearance of denatured antigens as these antigens, when a cell is dead, may be exposed to the recognition of the immune system. Some researchers have shown that some autoantibodies occurs in normal individual, most of them being against cytoskeleton proteins [Guilbert B. et al, 1982] [Stefansson K. et al, 1985]. In the same way, Babikian and colleagues described the detection of autoantibodies 'bands' on 'western blots' after incubation of sera from normal individuals with spinal cord extracts [Babikian V.L. et al, 1985]. In neurological diseases, proteins may be released in the circulation because of the disease process and these proteins could induce an humoral immune response.

However, the denaturation of the antigen before the SDS PAGE electrophoresis and electrotransfer may be an advantage as it may expose antigens which will be hidden otherwise. In the case of human brain vessels, antigens exposed on the intraluminal surface of the vessels are inaccessible on undisrupted purified vessels. These are the very antigens which are likely to be involved with a possible autoimmune reaction in MS. Therefore and despite its drawbacks, the 'western blotting technique was the only technique worth utilising to study possible autoantigens on brain vessels mainly because human brain endothelial cells in culture were lacking, otherwise, other techniques which may be more powerful - when well controlled - could have been used e.g the radioimmune precipitation technique. The same applies, to some extend, to myelin
as most of the myelin proteins in the myelin are hidden within the myelin sheath [Norton W.T., 1984].

a/ Discussion of the result of the western blotting assay with the purified vessels as antigens.

Only one group has reported that MS patients and patients suffering from HTLV1-associated myelopathy have autoantibodies to endothelial cells [Tsakuda N. et al, 1989] [Tanaka Y. et al, 1987]. However these particular observations were based on ELISA tests using rat endothelial cells as antigen and with low dilution (1/10) of the tested sera. Thus these observations needed to be complemented by other approaches and we decided to use the Western blotting technique to investigate the prevalence of autoantibodies to human brain antigens taking into account the limits of both methods.

Taking into account the limitation of the 'western blotting' technique discussed above, there was nevertheless no strong evidence in the present study that patients suffering from MS are more prone to develop autoantibodies against the BBB than other neurological controls. There is however a small proportion of individuals (MS and neurological controls) who possess autoantibodies against either myelin or vessels which can be visualised by the 'western blotting' technique. The significance and relevance of these autoantibodies are still unknown. The reaction seen in some individuals may in fact be a consequence of a neurological disease rather than a cause of the disease. It appears that the proportion of individuals showing autoantibodies against brain vessels (or myelin) is much higher in the patients groups (MS or neurological controls) than in the normal group. This difference could also be explained by the younger age of the normal group in comparison with the age of the patients groups. It has been reported that autoantibodies are more prevalent in the older population. However, the only normal individual who showed autoantibody against the brain vessels was only 25 years of age and there was no correlation with age among the neurological patients (MS and others) who showed autoantibodies against the brain vessels: the mean age was 45.5 years - range: 29-67 (n= 14).

Although the numbers are very small, it appears that the number of neurological patients suffering from epilepsy or migraine/headache and with autoantibodies to brain vessels detected on 'western blotting' was increased: epilepsy (20 %), migraine/headache (40%). Some have suggested that disruption of
the blood-brain barrier occurs in epilepsy [Bolwig T.G. et al, 1988][Cornford E.M et al, 1986]. Further studies on the prevalence on autoantibodies to brain vessels in patients suffering from epilepsy and in patients suffering from migraine/headache are necessary.

Unlike the myelin, background reactivity against human brain vessels was not observed with human sera and thus the reactivity observed in some individuals (patients and controls) seems genuine. However, a reliable human brain vessel-antiserum was lacking in this study which may have made these results more interesting as a positive control could have been used in each experiments. An attempt to immunise rabbits with whole human brain vessels resulted in an antisera with little reactivity. The antisera recognised two protein one of 22 kd and one of 43 kd protein. This may be due to the immunisation procedure that we adopted as we immunised the rabbits with whole purified human brain vessels in complete Freund's adjuvant. Critical antigens may have been hidden or the human brain vessel antigens may not have been 'foreign' enough for the immune system of the rabbits.

On the other hand, these autoantibodies directed to brain vessels in some neurological patients may be related to vessels specific components. Although little is known about the molecular structure of the BBB, some interesting features have already been uncovered and some proteins related to the BBB partially characterised. The exact location of the proteins detected by autoantibodies on 'western blotting' is not known. It has been shown that the protein composition of the luminal membrane is different from that of the antiluminal membrane of the brain vessels [Lidinsky W.A. et al, 1983]. If autoantibodies to brain vessels are important in the aetiopathogenesis of MS, it is likely (but not necessary) that they will recognise antigens situated at the luminal side of the brain vessels. W. M. Pardridge and colleagues raised an antisera against a 46 Kd protein which seemed to be specific for bovine brain capillaries [Pardridge W.M. et al, 1986]. In the same molecular weight region, a inducible BBB specific molecule called HT7 of around 45-52 Kd has been characterised in chick brain microvessels [Seulberger H. et al, 1990]. Sternberger N.H. and Sternberger raised a monoclonal antibody named anti-endothelial-barrier-antigen (EBA) which appeared to be specific for brain vessels including optic nerve, spinal cord and retina. The monoclonal reacted also with some vessels in the spleen and some cells in the epidermis of the
skin and recognised a 30/25/23.5 Kd triplet [Stemberger N. et al, 1987]. Other proteins have been located on the brain vessels e.g. transferrin receptor (a 95 kd dimer) [Jefferies W.A. et al, 1984], insulin receptor (127 kd protein) [Pardridge W.M. et al, 1985], P-glycoprotein [Gordon-Cardo C. et al, 1989] and proteins involved in glucose transport and other transport systems [Kalaria R.N. et al, 1988] [Pardridge W.M. et al, 1986]. Pardridge and colleagues have shown that low molecular weight proteins (14, 16, 17, 18 Kd) in isolated brain capillaries are histones [Pardridge W.M. et al, 1989]. Tsakuda and colleagues using the ELISA assay showed that sera from MS patients had antibodies directed to even lower molecular weight proteins (8, 11, 12.3 Kd) [Tsakuda N. et al, 1989]. However this observation needs to be confirmed. Some of the proteins detected by autoantibodies in our study (see table 1) may correspond to these characterised brain vessels proteins. They may also correspond to more common proteins like actin of molecular weight of 43 Kd.

Detection of autoantibodies to brain vessels in patients suffering from neurological diseases especially MS has been difficult because of the limitations of the assays used (immunofluorescence assay [Fillit H.M. et al, 1986], ELISA assay [Tsakuda N. et al, 1989], 'western blotting' technique). The histopathology of MS suggests that the MS lesions could be in part due to an immune response to brain vessels as part of an autoimmune reaction or because of a viral infection of the brain vessels. In epilepsy, autoantibodies to brain vessels may be associated with the disease and their aetiopathogenesis relevance needs to be examined. In another neurological disease namely senile dementia of Alzheimer's type, autoantibodies to brain vessels have been reported in the patients suffering from this disease [Fillit H.M. et al, 1986]. There is a need of further studies looking at the prevalence of autoantibodies to brain vessels in patients with neurological diseases and new assays should be devised to approach this question.

b/ Results with the myelin:

The results with the myelin as antigens were more difficult to interpret. Firstly there was an unspecific reaction when the myelin was only incubated with the second antibody (the anti-human IgG serum). This unspecific reaction was always observed around 95
This nonspecific reaction was not seen when the antigen was the human brain vessels. The reason for this unspecific reaction is not known; it may be that the region of the myelin concerned with this reaction has an endogenous peroxidase activity. Secondly, the pattern of reactivity - above the background - was more complex as most of the sera reacting with at least one or two proteins of the myelin preparation. This was observed also by Newcombe and colleagues who tested human sera by western blotting against CNS particulate fractions. Sera from normal individuals and sera from neurological patients reacted against a number of bands from the CNS particulate preparation [Newcombe J.I.A. et al, 1984]. In the case of the myelin, a positive antisera was available (kindly provided by Dr Eric Blair, Leeds). This antisera, raised against rat myelin, reacted against most of the human myelin proteins.

The occurrence of antibodies reacting to myelin in normal individuals is puzzling. They may be physiological as discussed above. The other possibility is that these antibodies are in fact low affinity antibodies which cross react with myelin but are of no physiological relevance.
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281


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