NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

THE UNIVERSITY OF ALBERTA

VIRUS INFECTION
AND
EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

by

LINXIAN WU 

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DRGREE OF Ph.D IN MEDICAL SCIENCES (VIROLOGY)

DEPARTMENT OF MEDICAL MICROBIOLOGY AND
INFECTIOUS DISEASES

EDMONTON, ALBERTA, CANADA

SPRING, 1989

ISBN 0-315-52969-5

Canada

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: LINXIAN WU

TITER OF THESIS: VIRUS INFECTION AND EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

DEGREE: Ph. D.

YEAR THIS DEGREE GRANTED: Spring, 1989

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed) C Wu Linxian

PERMANENT ADDRESS:

National Institute for the Control of Biological Products. Beijing. China

Date: February 16, 1989

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled VIRUS INFECTION AND EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS submitted by LINXIAN WU in partial fulfilment of the requirements for the degree of Ph.D in Medical Sciences (Virology).

.....

Supervisor

.....

.....

.....

Date: February 16, 1989

TO TWO WOMEN I LOVE

MY DEAR MOTHER - LAN-SHEN LIN

&

MY DEAR WIFE - XIAO-YING ZHUANG

ABSTRACT

In this study, a murine model combining a nonlethal Semliki forest virus (A7-SFV) infection and experimental allergic encephalomyelitis (EAE) was established. BALB/c mice were rendered susceptible by low dose irradiation. A7-SFV infection potentiated the rate of disease depending on the temporal relationship between the induction of EAE and the virus infection. The potentiation effect was mediated immunologically and disease could be transferred to naive recipients with the spleen or lymph node cells from neuroantigen primed and virus infected donors at day 10 or 12 after neuroantigen inoculation, i.e., day 3 or 5 after virus infection. The potentiation effect could also be achieved in neuroantigen primed mice by transfering spleen cells from virus infected animals. Infectious virus was not present in these cells. Cell depletion assays suggested that both T lymphocytes and macrophages were required for the potentiation effect. The upregulation of EAE in this model may also be related to the fluctuation in production of soluble immune modulators, such as IFNγ, IL-2 and IL-1. However, recombinant IFNγ did not potentiate EAE *in vivo per se*. *In vitro* and *in vivo* characterization of an intrinsic interaction between macrophages and virus showed that virus infection had a significant effect on the

functions of macrophages. A transient activation of macrophages by a restricted SFV infection led to an upregulated IL-1 production, Ia antigen expression, and subsequently, increased antigen presenting ability. Therefore, an aberrant regulation of virus infected macrophages may be a precipitating factor in the potentiation of EAE. This model supports the notion that virus infection can disturb the immunological balance and allow pre existing but suppressed autoreactive cells to express themselve and damage the host. The data suggest that this model is suitable for studying the synergistic effect of virus infection which is revelant for understanding of the new exacerbation of multiple sclerosis (MS). The results obtained from this model may also be applicable to studies of the immunopathogenesis of other autoimmune disease.

ACKNOWLEDGEMENT

I give special thanks to Dr. A. Salmi, for it is he who introduced me to the fascinating field of viral immunology.

I am grateful to Dr. R. G. Marusyk, for his active role in supervising my work.

I especially thank Drs. D. R. Green, M. S. Peppler, and K. C. Lee, members of my supervisory committee, for many invaluable and constructive suggestions.

I appreciate the help of Dr. M. Röyttä in histopathological examinations, and the help of Dr. K. Suryanarayana in performing RNA hybridizations.

I also thank Ms. B. Folkins, V. Jeffrey, and Mrs. L. Chui for technical assistance, Mrs. L. Lynklater for FACS assistance, and Ms. H. Esak for taking care of the animals.

This work was supported by the Alberta Heritage Foundation for Medical Research and the Multiple Sclerosis Society of Canada. I would like to avail myself of this opportunity to thank Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BBB: blood brain barrier

BMDM: bone marrow derived macrophages

BP: myelin basic protein

GP-BP - guinea pig MBP

BPL: β-propiolactone

ConA: concanavalin A

CNS: central nervous system

CPE: cytopathic effect

CTL: cytotoxic lymphocytes

DTH: delayed type hypersensitivity

EAE: experimental allergic encephalomyelitis

FACS: flow cytometry

FBS: fetal bovine serum

HSV-1: type 1 Herpes simplex virus

IL-1: interleukin-1

IL-2: interleukin-2

IFNα,β,γ: alpha, beta, gamma interferon, respectively

LDV: lactate dehydrogenase-elevating virus

LPS: lipopolysaccharide

MHV: murine hepatitis virus

m. o. i.: multiplicity of infection

MV: measles virus

MS: multiple sclerosis

MSCH: mouse spinal cord homogenate

OVA: chicken ovalbumin

PM: thioglycollate elicited peritoneal macrophages

PLP: proteolipid apoprotein

PFU: plaque forming unit

r-IFNγ: recombinant IFNγ

SFV: Semliki forest virus

A7-SFV - avirulent strain of SFV;

W-SFV: wild type strain of SFV.

$TCID_{50}$: 50% of tissue culture infectious dose

TMEV: Theiler's murine encephalomyelitis virus

VSV: vesicular stomatitis virus

INTRODUCTION

EAE as Animal Model of Demylination

Experimental autoimmune (allergic) encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) which can be elicited in different laboratory animal species with a single injection of neuroantigen, usually incorporated in an immunological adjuvant (1, 2). Clinical manifestation of paralysis usually occur after 11-21 days in a species-dependent manner after induction preceded by histological lesions of perivascular mononuclear cell infiltration in the CNS (1,2). The encephalitogenic activity of neuroantigen is organ-specific, rather than species-specific since autologous, homologous, or heterologous brain or spinal cord can be used to induce EAE. The antigenic activity of nervous tissue in experimental animals was first discovered by Brandt, Lewis, Rivers *et al.* (3 - 5). Monkeys were the first species employed, and the disease clearly resembled the acute encephalomyelitis in man (4, 5). Development of Freund-type adjuvants during the 1940s promoted the study and established the EAE as an autoimmune disease model (6). The Freund-type adjuvant consisted of killed mycobacteria suspended in paraffin oil and an emulsifying agent to assure creation of a stable water-in-oil emulsion after vigorous mixing of the nervous tissue homogenate and the

adjuvant. As reported in 1947, multiple laboratories demonstrated that monkeys, guinea pigs, and rabbits, developed acute encephalomyelitis following injection of brain or spinal cord homogenate combined with Freund's adjuvant (6 - 8, 11). Since then, EAE has become an established animal model for the study of allergic forms of inflammation and injury which affect the CNS of animal and man (1).

EAE is readily induced in guinea pigs, rats, rabbits and monkeys (1 - 8), but hitherto with greater difficulty in mice. Since many of the principles of contemporary immunology have been developed in mice, there are obvious advantages to studying EAE in this species. Mice were first used by Lee and Olitsky (9) to study EAE, and it was also clearly demonstrated that *Bordetella pertussis* vaccine is an important adjuvant for induction of EAE in mice, but not in other animal species (10). The effect of pertussis vaccine on EAE induction in mice may be due to multiple biological functions of pertussigen such as sensitizing to histamine and promotion of lymphocytosis (11, 12). It has also been shown that pertussigen increases vascular permeability in brain and vascular sensitivity to vasoactive amines which promote the development of EAE (13).

Susceptibility of Mice to EAE Induction

EAE is under genetic control in guinea pigs, rats

and mice, and genetically susceptible or resistant strains have been demonstrated (14 - 18). The disease could be induced in the SJL/J mouse strain, or in SJL/J crosses with various resistant strains such as NZB mouse strain (19 - 22). A great difference was observed in the sensitivity to EAE of the various F1 hybrids of SJL/J mice. Crossing the sensitive SJL/J strain with the resistant strains such as NZB, BALB/c or congenic strains BALB.B10 and BALB.C3H, led to fully susceptible hybrids. On the other hand, crossing SJL/J with other resistant strains such as C57BL/6J or DBA/2, led to hybrids with a low susceptibility to EAE. It was therefore postulated that in these resistant strains, the influence of the gene determining susceptibility to EAE is masked by the presence of naturally occuring suppressor cells (19 - 22). These suppressor cells may be sensitive to the treatment of low dose irradiation and cyclophosphamide (19). However, it has been suggested that EAE responsiveness was under the control of genes outside the H-2 complex. The F1 data do not show a unifactorial inheritance of EAE responsiveness (15 - 17). The F1 data imply a maternal factor, sex hormone, or sex-linked gene(s) that modifies EAE responsiveness. Therefore, the H-2 complex may modify the degree of EAE responsiveness (17). More likely, the susceptibility to induction of EAE may be controlled by two genes, one linked to the H-2 gene complex, and the other a non-H-2 linked locus. Three other genes may be associated with susceptibility to EAE

induction, two genes governing vascular sensitivity to vasoactive amines, and up to three 'histamine - sensitization responses' genes which are these are related to the use of pertussis as an adjuvant (17). It is known that pretreatment with cyclophosphamide, low dose whole body irradiation or high doses of pertussigen may render genetically resistant BALB/c mice susceptible to EAE induction (12, 19).

Pathogenesis of EAE

It is commonly accepted that EAE is a disease of delayed type hypersensitivity to encephalitogenic antigen (1), that is, sensitized T lymphocytes interact with encephalitogenic antigen in myelin. Circulating anti-brain antibody seems not play a significant role in the pathogenesis of EAE (1, 2). However, it has been found recently that *in vivo* administration of anti-myelin oligodendrocyte glycoprotein (MOG) monoclonal antibody can cause demyelination in mice (70). Therefore, the role of antibodies in the demyelination process requires further study. The central role of specific T cells is supported by some observations. For example, delayed-type cutaneous reactions to encephalitogens are demonstrable in animals developing EAE. The early lesion of EAE itself mainly consists of T lymphocytes and macrophages with a perivascular infiltration, and is independent of complement (1). More direct and convincing evidence has been produced

in a Lewis rat model for a major role of T cells in EAE (2). Thymectomized Lewis rats when reconstituted with bone marrow cells from syngeneic donors depleted of T cells by means of several days of thoracic duct drainage fail to develop EAE following sensitization with either guinea pig spinal cord or myelin basic protein neuroantigen, combined with adjuvant. If such rats are supplemented with normal Lewis thymocytes prior to sensitization to neuroantigen, practically all of the animals then develop typical EAE (23). Moreover, in both rat and mouse models (24, 25), anti-CD4 monoclonal antibody has been used to treat animals prior to disease induction or during disease induction and in both cases EAE is prevented (24, 25). In SJL/J mice (24), analysis of lymph node cell populations and antibody levels showed that animals treated with anti-CD4 antibody had a depletion of helper/inducer T cells and did not develop a humoral response to the administered rat antibody. Like Lewis rats which have recovered naturally from EAE, those mice treated with anti-CD4 MAb were even resistant to a secondary challenge with neuroantigen (25). This suggests that EAE is a DTH reaction mediated by CD4 molecule bearing T lymphocytes.

Immunological Effector Cells in EAE

The strongest evidence that EAE is a disease mediated by immune cells is the finding that it can be transferred with sensitized lymphoid cells into normal rats (26 - 30),

guinea pigs (31 - 33) and rabbits (34). The first successful transfer of EAE was demonstrated by Paterson (27) with injection of lymph node cells from actively sensitized donors into recipients pretreated neonatally with normal rat spleen cells. The purpose of the pretreatment with normal rat spleen cells was to extend the survival of donor cells in recipients. Later, a striking augmentation of severity of transferred EAE in the lightly irradiated recipients was found, possibly attributable to selective radiosensitivity of suppressor T cells(26, 28 - 30). In contrast to lymphoid cells, immune serum did not transfer this autoimmune disease nor did serum have any facilitating or inhibitory effect on the capacity of lymphoid cells to transfer EAE (28). It was also firmly established that the immune lymphoid cells responsible for transfer of EAE are T lymphocytes, since transfer was successful after passage of sensitized cells through anti-immunoglobulin columns and was abrogated following treatment with anti-Thy.1 serum and complement (28). Furthermore, it was also shown that a subset of T lymphocytes bearing Lyt.1 and I-A phenotypes is central to the transfer of EAE since pretreatment of cells with either anti-Ly.1 or anti-I-A monoclonal antibodies abolished the effect of transfer. Simultaneously, removal of I-E+ cells and both cytotoxic/suppressor and B cells had no effect on the disease outcome (36 - 43, 71).

Recently, the concept that the lesions in EAE are

produced by a classic DTH reaction was challenged by Sedgwick *et al.* (204). By adoptive transfer of EAE into irradiated or non-irradiated Lewis rats, they showed that the bulk of infiltrating cells in the CNS were superfluous to the induction of disease, as lethally irradiated recipients, despite having very few infiltrating cells in the CNS, acquired severe paralytic EAE. Disease in irradiated recipient animals was associated with substantial submeningeal hemorrhage in the spinal cord and brain stem and similar hemorrhages are found in recipients rendered leukopenic with cytotoxic drugs. Furthermore, clinical signs of EAE can be prevented by administration of anti-CD4 MAb. Therefore, acute EAE could be mediated by the direct action of very small numbers of activated CD4+ lymphocytes that infiltrate the CNS and produce their effects by inducing vascular damage (204).

EAE can be transferred directly to normal syngenic recipients with sensitized lymph node cells *in vivo* or with sensitized rat spleen cells that have been incubated *in vitro* with Concanavalin A (ConA) or encephalitogen (33, 35) in a mouse model. ConA and encephalitogen may be acting through different mechanisms in the activation of spleen cells from sensitized donors (37). Recent results indicate that both IL-1 and IL-2 are required for the activation of the effector cells that mediate this autoimmune response (38, 41). The effector cells are present in the T lymphoblast population enriched for the subset bearing the

CD4 surface marker (38, 42). In the rat model, the T helper cells are not the actual effectors within the recipients (38). Spleen cells from Lewis rats that have been immunized with guinea pig MBP in complete Freund's adjuvant can be activated during mixed lymphocyte reaction (MLR) to allogenic spleen cells and transfer EAE. This cell activation correlates with IL-2 production (43).

In 1981, EAE was first induced by adoptive transfer of lymph node cells primed *in vivo* with encephalitogen and incubated *in vitro* with encephalitogen in SJL/J mouse model (35). Furthermore, when the activated lymph node cells were treated before transfer with anti-Thy.1 or anti-Lyt.1 antibody and complement, neither clinical nor histological signs were observed in recipients. Therefore, these results provided the direct evidence that $Lyt1^+2^-$ T cells are responsible for the transfer of EAE in mice as well. Anti-Ia antibody also has been noted to both prevent and suppress active EAE in mice (47, 48).

Ben-Nun *et al.* (44) were the first who successfully isolated and grew rat T cell lines specifically reactive against guinea pig myelin basic protein (BP). These lines induced clinical and pathologic EAE in syngeneic rats 3 to 5 days after i.v. injection of neuroantigen (45). Activity of the line cells *in vivo* required prior activation with BP presented by histocompatible accessory cells (46). These T cell lines all express the T helper phenotype which provides confirmatory evidence that $CD4^+$ T lymphocytes play

a principal role in EAE induction.

Encephalitogens in EAE

Myelin basic protein (BP) is commonly regarded as a major constituent in CNS tissue with encephalitogenic activity (51-55). MBP occurs in three molecular forms in man and six in the mouse, encoded by a single gene on chromosome 18 in both species (1). There is a strong homology between MBP of different species. When combined with adjuvant, myelin basic protein prepared from bovine, guinea pig, or human CNS tissue can induce EAE in multiple species (1). Specific amino acid residues and sequences which determine encephalitogenic activity for different species of animal hosts have also been identified (56 - 58). For example, Lewis rat T cell lines and clones reactive against GP-BP responded selectively to the major encephalitogenic region of the GP-BP or rat BP molecule residues 68-88, but responded poorly to bovine or human BP molecules that have amino acid insertions in the encephalitogenic sequence (61). GP-BP was shown also to be encephalitogenic in SJL/J mice by direct challenge and in experiments in which an adoptive transfer system was employed (62). The encephalitogenic activity resided in the C terminal half of the molecule (62). Therefore the encephalitogenic activity of GP-BP could be different in different species and even strains.

Another major protein component of CNS myelin,

proteolipid apoprotein (PLP), is an encephalitogen, and can induce EAE in mice (59, 60, 64 - 66). PLP is encoded by the X chromosome. The 30 KDa molecule consists of highly conserved sequences with alterating hydrophilic and hydrophobic regions. A 3D model has been developed defining transmembrane segments T1, T2, and T3, as well as cytoplasmic and extracellular segments. Some of these, notably peptides 142 - 150 and 209 - 217, show regions of homology with BP (67). The encephalitogenicity of PLP has long be debatable because of possible contamination with BP. Hashim *et al.* (1980) claimed that PLP free of BP could induce acute EAE in guinea pigs (63). On the other hand chronic relapsing EAE was induced in guinea pigs and rabbits and rats by PLP (65, 66, 68). DM-20, a protein component of PLP was shown to induce EAE in genetically resistant BALB/c mice (67). However, it may not apply universally in BALB/c mouse strain since variability among substrains of BALB/c mice has been reported (67). Myelin lipids do not act synergistically with a non-encephalitogenic dose of MBP to induce EAE, but they induce immunological changes and potentiate the immune response to BP (69). It has been suggested that other autoimmune components might also be the target of this demyelination (70). Myelin oligodendrocyte glycoprotein (MOG) may be another important target in this autoimmune attack since antibody to MOG has been demonstrated to accelerate clinical and pathological changes on both acute and

chronical EAE in SJL mice (70). Different mouse substrains may respond to different encephalitogens (67). However, there is no information concerning the mechanism of this difference among BALB/c substrains (71).

Clinical Manifestations of EAE

Ataxic gait, paresis or paralysis of both hind limbs, fecal impaction and urinary retention due to autonomic nervous system dysfunction from spinal cord injury are the common clinical manifestations of EAE (72 - 74). The latent period of disease onset vary among different species, but the clinical neurological signs usually appear within 10-21 days after neuroantigen inoculation (1). In SJL/J mice, disease onset occurs about 11-13 days after sensitization. However, type and dose of encephalitogen, type of adjuvant, and route of inoculation influence the picture and course of EAE (1). It has also been discovered that the neurological signs correlate well with the demyelination process in CNS (75).

Pathology and Immunopathology of EAE

By light microscopy, the pathognomonic microscopic lesions of EAE consist of sharply circumscribed focal areas of perivascular inflammation within the brain and spinal cord, affecting the white matter more than the grey matter (1, 2, 51). The initial lesions are perivascular and grow by radial enlargement and confluence. The inflammation

induces edema, formation of perivascular cuffs, and infiltration of the parenchyma with both T and B lymphocytes and large numbers of macrophages. The inflammatory cells are predominantly mononuclear macrophage-like cells, and a small number of lymphocytes and a few plasma cells. Reactive changes involving CNS glial cells surrounding the focal vasculitis consist of a proliferation of microglia and astrocytes (1).

An important component of the EAE lesion is the demyelination around nerve fibers running through or near to the cellular infiltrates, a hallmark of this group of diseases. Swelling, fragmentation or complete dissolution of the myelin sheaths may be observed (76). By both light and electron microscopic examination, it seems clear that demyelination occurs only in areas where infiltrating mononuclear cells are or have been present (76). The demyelination involves both receptor mediated endocytosis by macrophages (anti-myelin antibody appears to serve as ligand) and extracellular lysis of unknown mechanism. Candidates for mediators of demyelination are macrophage enzymes, complement, endogenous proteases and free radicals. Nevertheless, the detailed mechanism of the demyelination is still unknown (1, 2). It is also unclear whether the initial encounter of the circulating, sensitized T cells with CNS myelin takes place at the luminar surface of vascular endothelium at the foot processes of astrocytes impinging on the perivascular space

or on glial cells in the parenchyma. BP appears to be present at all three sites by immunohistochemical criteria (1).

Adams dissected the sequence of inflammatory events and showed that depletion of myelin was preceded by invasion of the tissue by small lymphocytes which later gave way to large mononuclear cells (140). Development of monoclonal antibody technology provides for more precise characterization of these phenomena. With monoclonal antibodies to surface molecules of different cell populations, it has been shown that T cells and some IgG enter the CNS and migrate into the white matter parenchyma as early as 5 days postinoculation (p. i.) of the neuroantigen. By 10 days PI, immediately prior signs, B cells and macrophages appear, but these remain in the vicinity of the perivascular space. These patterns suggest that T and B cells have different migration abilities (2). Immunopathological studies show that T cells appear to be involved in lesion growth and have a parenchymal distribution, whereas suppressor/cytotoxic T cells seem to remain in perivascular locations around the perimeter of the lesion. Ia+ cells are always present and are associated with active breakdown of myelin (79, 81).

Perivascular infiltration of inflammatory cells, and demyelinated plaques in the central nervous system are also typical for patients with multiple sclerosis (MS), suggesting that autoimmune demyelination is a central

pathological event in MS. The progress of myelin destruction in EAE and MS has many features in common (2). It is also accepted that the exacerbations of MS correlate with overall decreases in circulating T cell levels and that at least some of these might be due to lymphocyte migration to the CNS. Other pathogenetic features of the immunocytochemical localization of released myelin component, axonal sparing and sclerotic, demyelinated lesions are also found in MS as they are in EAE (2). Both the acute and especially chronic relapsing EAE, have become good models for studies of acute MS, and pathogenesis and therapy of chronic MS (1, 2, 77).

The possible importance of MHC - restricted recognition events is suggested by the simultaneous presence of Ia antigens on vascular endothelium within the MS or EAE lesions and on activated astrocytes, as well as on the infiltrating macrophages and B cells. Macrophages are of great importance in both MS and EAE which is largely or entirely inhibited by doses of silica that wipe out the macrophage populations (38). Recently, immunopathological studies in chronic relapsing EAE in the mouse support the possibility of local antigen presention on endothelial and astroglial cells and an essential involvement of CD4+ T cells in CNS lesion formation. CD4+ cells predominate early and CD8+ cells in older lesions (77). Production of IL-1, IL-2 and IFNγ have been shown by immunohistochemistry and radioimmunoassay within the lesions and CSF of EAE animals

(78). These findings correlate well with studies on acute and chronic MS lesions (77, 78).

Virus Infection and MS

Virus infection in late childhood may be a precipitating etiologic factor of MS (80). Accumulated epidemiologic evidence, i.e. from twin studies, supports this notion (168). It has been postulated that an antecedent subclinical pathologic event occurs within the CNS due to a viral infection whereby CNS tissue damage and a breach in the blood brain barrier (BBB) may lead to sensitization of T and B cells to myelin components. Extrinsic factors, for example, a secondary infection, may break the threshhold of tolerance to the autoantigens. Antigens presented on the cell surface in CNS are recognized by specific T cells that produce lymphokines and cause a breach in BBB. Cells then enter the CNS tissue, other T cells are recruited nonspecifically, and a cascade of effector mechanisms is activated which culminates in local antibody production and myelin phagocytosis by macrophages. The cumulative effects of primary demyelination, axonal damage, vascular fibrosis, glial hypertrophy, and oligodendroglial depletion contribute an area of demyelination-plaque.

Persistent virus infection of nerve tissue may provide a continuing antigenic stimulus resulting in inflammation and demyelination, as shown in model studies

of Theiler's murine encephalomyelitis virus and a number of other viruses (245). Elevated production of antibodies to some viruses, especially measles virus, was demonstrated as a general characteristic of MS patients (82, 83). Significantly increased CSF/serum ratios of measles virus hemagglutination inhibition (HI) or nucleocapsid complement fixing (CF) antibodies were found in a large number of MS patients. Therefore, it was suggested that measles antibodies may be produced in the central nervous system by activated latent virus infections in some patients of MS (82). Recently, it was shown that class II restricted measles virus specific CTL are functionally impaired in MS patients (84). However, despite extensive studies, no clear-cut connection between virus infection and MS has been established (85, 86). Even though numerous attempts have been made to isolate viruses from MS brains and different strains have been obtained, no single virus has been consistently isolated (88). The recent development of *in situ* nucleic acid hybridization of viral genetic material support the above fact. The possible relationship between viruses and MS may not be specific, but instead, nonspecific (88).

It has also been the opinion of many clinicians dealing with MS patients that acute exacerbations of MS are often preceeded by an infection. Recent evidence strongly suggests that new exacerbations of MS are frequently connected with viral infections (87). Therefore, the role

of viruses in etiology and pathogenesis of MS is still favored. Nevertheless, the mechanism of the potentiation effect by virus infection is entirely unknown. An adequate animal model seems to be necessary to search for the answer (92, 99 - 103).

Virus Infection and EAE

Studies of a possible relationship between virus infection and autoimmune demyelinating disease are carried out from two different aspects. First, some viruses are found to cause demyelination in susceptible animals which resemble the lesion in brains of MS patients (89). Strain JHM of murine coronavirus has been thoroughly studied, and the demyelination in naive mice is inducible by T lymphocytes from virus infected mice (90, 91). Therefore, the nature of this virus induced demyelination is immunologically mediated (90). Theiler's murine encephalomyelitis virus can infect oligodendrocytes in the CNS, and virus infected cells may become a target of an immune attack (92 - 94). A mutant of Semliki forest virus, strain A7, has been found to cause demyelination in some mouse strains, and the effect can also be transferred with CD4+ T lymphocytes to naive animals (95 - 98). These models may support the idea that virus infection could be responsible for the disease onset of MS.

Secondly, other studies emphasize the synergistic effect of virus infection on EAE development which may help

understand the fact that virus infection could be an exacerbating factor in MS development. Persistence of measles virus in hamster brain has been shown to render this unsusceptible animal species susceptible to EAE induction (99). In this study, adult hamsters that have clinically recovered from acute encephalitis induced by prior intracerebral injection of measles virus were challenged with neuroantigen plus adjuvant. Such hamsters, which had a high likelihood of carrying persistent measles virus in the CNS, exhibited a significantly higher incidence of EAE following challenge as compared with simultanenously challenged but previously uninfected littermates. The occurrence of EAE in hamsters previously injected with heat-inactivated virus was not potentiated. Virus infection was required for this potentiation effect. Furthermore, presence of cell free or activated measles virus could not be demonstrated and suggested that this potentiation effect is not caused by activation of persistent measles virus (99). However, intraperitoneal inoculation of the same virus did not have an effect (100), so the route of virus infection was critical in this accelerating process, and altered patterns of EAE in infected animal may be due to virus induced changes in the target tissue.

Lactate dehydrogenase elevating virus (LDV) has also a modulating effect in EAE(101, 102). Infection of mice with LDV is non-lethal and induces life long viremia (104).

Macrophages appear to be the principal but not necessarily the only target cells (109 - 114), and several immunologic perturbations such as depression of cell mediated responses (109 - 114) have been reported. When SJL/J mice are infected intraperitoneally with LDV before inoculation with neuroantigen, the incidence of EAE is significantly reduced (101). The time of disease onset was also delayed in the infected group. However, when mice are infected six days after neuroantigen inoculation the incidence of disease and the day of disease onset is the same as in uninfected mice (102). In a C57BR/cdJ mice model, Stroop *et al.* have investigated the effect of LDV infection one week before or after inoculation of neuroantigen, and shown that both clinical and histological signs of EAE are accelerated (102). Type 1 Herpes simplex virus (HSV-1), the only DNA virus used in EAE studies until now, can enhance the development of EAE in hamsters (103). In the study, 50% of rats given two intracerebral injections of HSV-1, one before and one after induction of EAE, showed clinical and pathologic evidence of recently exacerbated EAE 16 days after the second HSV-1 injection, and no EAE is observed in mock inoculated animals (103). Recently, Semliki forest virus has been used to predispose and render a genetically resistant mouse strain, B10.A, susceptible to the EAE induction (115). When adult B10.A mice were inoculated systemically with 10^4 PFU of strain A7-SFV they developed a transient encephalomyelitis and sporadic mild symptoms of

paralysis with cerebellar demyelination from which they recovered. Such recovered mice were found to develop signs characteristic of EAE 2 to 8 wk after either immunization with BP or after receiving 1 to 2 x 10^7 lymph node cells from BP-primed syngeneic donors. These two methods of disease induction were unsuccessful when applied to normal B6 mice or those previously inoculated with noninfectious virus (115). It is evident that virus infection can have a synergistic effect on EAE onset and development. However, lack of knowledge of possible effects on the lymphoid cell system at the cellular and molecular level by virus infection make the interpretation of these phenomena difficult.

Hypotheses of the Potentiation Effect of Virus Infection on EAE

Some hypotheses have been postulated to elucidate the potentiation effect of virus infection and are briefly described below:

1. Antigen similarity (molecular mimicry)

Fujinami and Oldstone have recently developed and established the concept of molecular mimicry between viral antigens and tissue components as a mechanism of autoimmunization (167). They have identified a six amino acid peptide in hepatitis B virus polymerase which is identical with an MBP sequence known to be encephalitogenic in rabbits. A synthetic 8 residue peptide, incorporating

this hexapeptide induced both antibody and T cells reactive with whole BP and even produced mild EAE in rabbits (167).

PLP also has regions of partial homology with a variety of antigens such as HIV-1, measles virus, and Epstein Barr virus (245). Cross reactivity of glycolipid between enveloped viruses and CNS myelin has been suggested (105, 106). Certain enveloped viruses eg., MHV and SFV, can directly induce a T cell mediated demyelination in CNS (105). It could be that the demyelination is caused by T cells reacting against viral and/or self antigens on the surface of CNS cells. Virions could present the CNS glycolipids in their envelopes in an actively antigenic form to the immune system and trigger CNS autoimmunity (105).

It has been found by immunoelectron microscopy that a monoclonal antibody raised against CNS myelin reacts against not only a glycolipid component of myelin, but also labels brain derived SFV and reacts with this virus in an ELISA (106). Therefore, an infection with a neurotropic enveloped virus can generate antibodies cross-reactive with a glycolipid component of CNS myelin, and this glycolipid is closely related, or identical to, a glycolipid present in the envelope of SFV. Such glycolipids, normally haptens, in association with the proteins of the viral envelope could be antigenic and a susceptible individual could induce an anti-glycolipid autoimmune response to CNS cells, resulting in demyelination. This cross reactivity may also

play a role in synergistic effect of virus infection on EAE.

2. Adjuvant effect of virus infection .

From an immunobiological standpoint, double stranded RNA of either microbial or synthetic origin has a powerful capacity to potentiate immunological responses to diverse antigenic stimuli (107). The adjuvant activity of such double stranded RNA has been shown to embrace both cell-mediated immune responses and antibody production (107). Cone and Johnson (108) have provided evidence that the adjuvanticity of double stranded RNA may reside in their capacity to expand T cell populations engaged in responding to antigenic stimulation which may be mediated by high level IFN production stimulated by double stranded RNA (108). Therefore, it is possible that replication of RNA viruses, e. g., lactate dehydrogenase elevating virus, can have an adjuvant effect and enhance autoimmunity (102).

3. Deletion of Ia antigen bearing macrophages

Administration of monoclonal anti-Ia antibody to experimental animals reduces the incidence of various autoimmune disease (47, 48). Although the mechanisms are not fully understood, it is assumed that elimination of antigen presenting cells such as macrophages, dendritic cells and Langerhans cells interfere with the autoimmune process. LDV selectively infects and destroys Ia antigen positive macrophages (109 - 114). The proportion of Ia positive peritoneal macrophages was found to be lower in

LDV persistently infected mice (112). Thus, although LDV conceivably has an effect on Ia positive T cells, it was suggested that the most likely explanation for decreased incidence and severity of EAE in infected mice can be due to a decreased antigen presenting capacity (101).

4. Lytic virus infection in CNS tissue.

Infection of rats with Theiler's murine encephalomyelitis virus produces a late onset demyelinating encephalomyelitis due to a lytic replication of the virus in oligodendrocytes (93). It is also suggested that replication of LDV or some other viruses in the CNS may cause the release of neural antigens, in a manner similar to TMEV infection (92, 93, 94). These antigens may then participate in the EAE process to hasten and worsen the CNS lesions observed, perhaps through production of effector T cells.

Nevertheless, there is little experimental evidence for the above speculations in animal models used for studing the synergistic effect of virus infection in EAE. Two very critical issues have yet to be answered in cases of synergistic effect of viral infection on autoimmune disease development: First, is it virologically mediated or immunologically mediated? Hypotheses 1 and 4 favor that the synergistic effect of virus infection on EAE development could be mediated virologically. And the hypotheses 2 and 3 suggest an immunological basis for this synergistic effect. Second, if it is mediated either immunologically or

virologically, what is the mechanism? None of the studies mentioned above provide direct evidence in support of any hypothesis. Virus infection is a complicated event and its modulating effect *in vivo* may not be simply explained by a single mechanism. Therefore, detailed virological and immunological studies with an animal model are necessary to give important clues for the understanding of the etiology and pathogenesis of multiple sclerosis, especially on onset and new exacerbations.

Virus, CNS Cells, Cytokines and EAE

Since T lymphocytes recognize Ia antigens in association with other specific antigens, the cell type expressing Ia antigens in the normal nervous system and during the initiation of disease process may be responsible for the local activation and control of T cells. Microglial cells, a macrophage-like cell type in the CNS, has been shown to be responsible for producing interleukin-1 (IL-1) locally and may participate in the antigen presenting process as well (118). Bone marrow origin of microglial cells has been suggested, but circulating monocytes may also be localized and mature in the CNS (118). Astrocytes and endothelial cells, normally do not express Ia antigens, but can express Ia antigens under stimulation, for example, when stimulated by IFNγ (119). Furthermore, both primary astrocytes and astrocyte cell lines have been shown to present antigen to myelin basic protein specific T

cell lines when astrocytes are activated to express Ia antigens by IFNγ treatment or cocultivated with T cell lines (119). Astrocytes can also proliferate in the presence of mitogen- or antigen- stimulated T cell supernatants (120), or supernatants from human T lymphotropic virus (HTLV-1) transformed T cells (121), and purified human IL-1 (122, 123). Oligodendrocytes, myelin sheath forming cells in brain, can proliferate and differentiate when incubated with supernatants from mitogen activated or HTLV-1 transformed T cells (124). In particular, oligodendrocytes can be stimulated to differentiate by recombinant IL-2 and a newly defined factor - glial growth promoting factor (GGPF) released by activated T lymphocytes (124). Therefore, cytokines, including IL-1, IL-2, IFNγ, etc., released by activated immune cells, may have a critical role in initiating an inflammatory reaction of an autoimmune CNS disease. Up-regulation by IFNγ may permit expression of rare MHC molecules, with an enhanced presentation of unusual autoantigen epitopes and an enhanced probability of autologous mixed lymphocyte reaction (126).

The JHM strain of murine coronavirus can induce Ia antigen expression in both macrophages and astrocytes. The induction is not mediated by soluble factor but, instead, a physical interaction between viral glycoprotein E2 and receptors on astrocytes is needed. The kinetics of virus-induced Ia expression is slower than IFNγ induced Ia

antigen expression (119). Therefore, this implies that virus which can reach the CNS before any inflammatory cells and cytokines could initiate a localized immunological reaction. Particularly, the JHM virus can induce an EAE like disease in susceptible animal strains (125). No such information is available to explain the synergistic effect of virus infection on EAE development. Albeit, aberrant immune regulation after virus infection could lead to an autoimmune disease as suggested in autoimmune thyroiditis (126).

Virology and Pathogenesis of Semliki Forest Virus (SFV)

Virus is a major component in an animal model used to study the synergistic effect of virus infection on autoimmunity. A non-lethal mutant of Semliki forest virus (A7-SFV), selected by spontaneous attenuation through intracerebral passages in mice and originally designated A774 (127), was used in this project. SFV, an enveloped animal virus belonging to the alphavirus group of the family Togaviridae, causes natural infection in mice. Wild type strain of SFV (W-SFV) rapidly kills the mice as a consequence of meningoencephalitis (119). Stable, attenuated mutants of SFV have been isolated and used in pathogenetic studies (127, 128). The structural properties, the biosynthesis, and the general features of SFV have been well characterized (127 - 139).

SFV consists of a spherical nucleocapsid containing a

single-stranded RNA molecule of 12,700 nucleotides long and 240 copies of a single protein (142, 143). The nucleocapsid is surrounded by a lipid bilayer membrane with glycoprotein spikes (144). Each of these spikes consists of three glycopolypeptides, with apparent molecular weight of $4.8x10^4$, $5.1x10^4$, and $1x10^4$, and designated E1, E2, E3 respectively (142, 145). The spike proteins are anchored by hydrophobic segments in the lipid bilayer and behave as ligands for receptors on the cells (146). In some susceptible cells, infection proceeds rapidly (147), penetration occurring within the first hour after addition of the virus. The viral RNA synthesis is detectable after 1.5 h and after 2.5 h the first progeny viruses appear in the medium (147).

SFV enters the cell evidently by receptor mediated endocytosis (RME), and fusion of the viral membrane with the vascular membrane is required for releasing of the viral genome into the cytoplasmic compartment (148, 149). Class I MHC molecules are used by SFV as receptors in mamalian cells(148, 149).

The replication of SFV in various human lymphoblastoid cell lines has been studied (139). SFV can replicate in most of the cell lines, but in certain cells the replication is severely suppressed. The suppression occurs after virus is absorbed to cells but at which stage the viral replication is inhibited is still unknown (139). The infection of primary macrophages is a controversial

topic (150, 151). Van der Groen *et al.* demonstrate that SFV is able to replicate in murine peritoneal macrophages *in vivo*, and the virus replication is enhanced in macrophages induced by proteose peptone (150). However, Kraaijeveld *et al.* are unable to detect any infectious virus in thioglycollate stimulated peritoneal cells *in vivo* (151). Further, the effect of virus infection in immune cells, especially macrophages, is entirely unknown. Semliki forest virus can also persistently infect murine L929 cells. The persistently infected cells are resistant to superinfection by both homologous and heterologous virus. It is suggested that interferon is responsible for the establishment of this persistent infection, since a similar persistent infection can be established by pretreatment of uninfected cells with mouse interferon (152). Atkinson *et al.* have demonstrated the establishment of persistent infection in mouse brains by co-inoculation with defective interfering particles (153).

The first isolation of Semliki forest virus was from a pool of 130 female mosquitoes (*Aedes abnormalis Theobald*) caught in Bwamba, Uganda in 1942 (154). Several other independent isolations have been made of agents identified serologically as SFV (155, 156). Both virulent and avirulent strains have been identified (127). Mice up to two weeks of age are infected lethally by any strain of SFV administered by any route. Between 15 and 20 days of age a rapid change occurs, resulting in almost totally resistant

(>10^7 PFU/LD50) to lethal infection by A7 - a strain used in this study (127). Age-dependent and strain- related differences in mouse susceptibility to SFV were also characterized by Flemin in 1977 (157). A7 does not kill mice and replication becomes undetectable at day 4 or 5 post infection, and there is no evidence of replication in the spleen after the peak of viremia. The virulent strains generally cause death before antibody can be detected in brain tissue; the avirulent strain causes an infection that is neuroinvasive, but infectivity increases less rapidly in the brain and allows antibody to intervene before clinical signs are apparent. This could be the differences between virulent and avirulent strains in terms of pathogenesis (157). Some neurovirulent mutants have been isolated by mutagenesis (158). These mutants (i.e. M9) produce paralysis in 35% of infected mice, and 8% died. Demyelination occurs in 95% of the surviving mice and is associated with the destruction of oligodendrocytes. The virulence of the virulent strain is due to its ability to destroy both neurons and oligodendrocytes, whereas the demyelination produced by mutant M9 is due to the destruction of oligodendrocytes alone (159).

It has also been noted that strain A7 does not kill either neurons or oligodendrocytes (130). Infection of adult mice with strain A7 induces demyelination in a small number of animals (160). Maximum demyelination occurs between days 14 - 21 when virus has been cleared from the

brain (160). It is suspected that A7 induced demyelination is not the direct effect of virus replication, and the interaction between lymphocytes and other cells (especially macrophages) seen in the lesions suggests the presence of some specific immunological interactions between lymphocytes, macrophages and glial cells (130). Strain A7 of SFV can persistently exist in brains of nu/nu mice for a long time but not in immunocompetent mice. This long term persistence of A7 results in no obvious destruction of central nervous system cells, and no demyelination can be found. When sensitized spleen cells from mice infected with A7 are transferred to A7 infected nu/nu mice, virus is eliminated and demyelination induced (131, 161). These data suggests that A7 induced demyelination is immunologically mediated.

Immunologic responses of mice infected with SFV have also been extensively characterized. Both neutralizing and non-neutralizing monoclonal antibodies to the E2 glycoprotein of SFV can protect mice from lethal encephalitis (133). It has also been demonstrated that after SFV infection of BALB/c mice highly cytotoxic activated macrophages, not specific for the infecting virus, appear at day 1, peak on day 2 to 3, and disappear within a week. Specifically sensitized T cells (CTL) appear around day 3, peak on day 6, and disappear within a month (136). Delayed type hypersensitivity (DTH) appears in mice after SFV infection and local transfer of DTH by peritoneal

exudate cells has been shown (134). Circulating SFV may infect capillary endothelial cells and eventually reach the brain. In this replication process, interferons can be induced, IFNα,β produced early, and IFNγ later (135, 162). SFV replication is sensitive to the treatment of IFNs (162). It has been shown that β-propiolactone-inactivated preparation of SFV (BPL-SFV) can stimulate SFV specific T cells to release IFNγ, and released IFNγ may have a feedback effect on expression of class I and class II antigen expression of brain cells (163, 164). However, the relevance of this effect with A7 induced demyelination is not known.

Objectives of This Project

Accumulated evidence suggests that MS may be initiated by infection with a common respiratory or exanthematous virus in late childhood (168), and a recently published study by W. Sibley *et al.* strongly suggests a direct involvement of virus infection in a new exacerbation of MS (87). Studies of the relationship between virus infection and MS demand a suitable animal model. However, none of the models have been satisfactory in elucidating the mechanism of virus infection on the onset or development of demyelination. T cell tolerance, mediated by antigen alone or indirectly by suppressor cells, has been the most common explanation (165, 166). However, it may also been possible that lack of autoreactivity towards

cell-surface antigens is due to lack of effective autoantigen presentation. Therefore virus infection may stimulate the release of soluble immune modulators from immune cells, i.e. IFNs, ILs, etc., and up-regulating autoantigen presenting process which may lead to an aberrant immune regulation and trigger autoimmunity. This sequence of events is possible as even normal people may have autoantigen specific cells which can react with BP (245).

In this project, I intended to establish a mouse model for studying the following questions which may not be feasibly carried out in clinical studies:

1. What is the effect of A7-SFV infection on the onset and development of EAE in mice? Does this represent a mouse model to study the potentiation effect of virus infection on EAE onset?

2. If the potentiation effect can be observed, is the effect mediated virologically or immunologically?

3. What is the cellular basis of the potentiation effect?

4. What is the effect of a restricted infection of A7-SFV in murine macrophages on functions of immune cells and the relevance to potentiation effect?

5. Can some mechanisms of autoimmunity be explained by this model?

MATERIALS AND METHODS

Viruses

An avirulent strain of Semliki forest virus (A7-SFV), was obtained from Dr. H. E. Webb (Neurology Unit, Department of Neurology, Rayne Institute, St.Thomas' Hospital, London, U.K.) and a virulent strain of SFV (W-SFV) was obtained from Dr. Sirkka Keränen (Department of Gene Technology, University of Helsinki, Finland). The virus was grown in a BALB/c brain cell line (MBA-1) and titrated with a standard plaque assay in another BALB/c mouse brain cell line (MBA-13) established in this laboratory. The stock virus with a titer of 10^7 to 10^8 p.f.u./ml was aliquoted and stored at -70° C until used. Measles virus (strains Halle, Lec, and a temperature-sensitive mutant, ts38), Herpes simplex virus type 1, human adenovirus type 3, poliovirus type 1 were kind gift of Dr. R. G. Marusyk and Mrs. L. Chui of this Department.

Cells

MBA-1, MBA-13 and L929 cell lines were grown at 37° C in modified Eagle's minimal essential medium (Mediatech., Washington, D.C.) supplemented with 1% glutamine, 200 I.U./ml penicillin-G, 200 µg/ml streptomycin and 3% heat inactivated fetal bovine serum (FBS), adjusted to pH 7.4 with sodium bicarbonate. IL-2 dependent CTL line cells (a

kind gift of Dr. D. R. Green, Department of Immunology, University of Alberta) were grown in RPMI medium containing 200 I.U./ml penicillin-G, 200 µg/ml streptomycin, $5x10^{-5}$ M 2-mercaptoethanol, 2% concentrated culture supernatant of EL-4 cells as a sourse of IL-2 (a gift of Dr. K. C. Lee, Department of Immunology, University of Alberta) and 10% FBS. Cell lines were routinely examined for mycoplasma contamination with a standard isolation procedure done in the Mycoplasma Laboratory in this Department. No mycoplasma was detected in any of cell lines.

Mice

Six to eight week old female BALB/c mice were obtained from the Health Sciences Laboratory Animal Services, University of Alberta, or Charles River Laboratories, St. Constant, Quebec. Six to eight week old SJL/J mice were purchased from Jackson Laboratory, Bar Harbor, Maine. All animals were housed in plastic cages at 22° C on wood chip bedding and offered food pellets and water *ad libitum*. Animals infected with the A7-SFV strain of SFV were housed in separate but otherwise identical quarters.

Preparation of Mouse Spinal Cord Homogenate (MSCH)

BALB/c mice approximately 3 months of age and SJL/J mice 8 months of age were sacrificed by cervical dislocation, spinal cords removed by flushing and immersed in sterile phosphate buffered saline (PBS, pH 7.0) on ice. The tissue

was homogenized in sterile distilled water using Broeck homogenizers to give a 40% (w/v) mixture which was lyophilized, ground to a fine powder and stored in a desiccator at -20° C until used.

Adjuvants

Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), *M. tuberculosis* H37RA and *M. butyricum* were purchased from Difco Labotories, Detroit, Michigan, U.S.A.. *B. pertussis* bacteria were a gift of Dr. M. S. Peppler, Department of Medical Microbiology and Infectious Diseases, University of Alberta.

Irradiation of BALB/c mice

BALB/c mice were exposed to total body irradiation in a Gammacell 40 X-ray machine (Atomic Energy of Canada Ltd.). In inducing EAE in BALB/c mouse strain, mice were irradiated with 350 R 2 days before neuroantigen inoculation. When inducing EAE by cell transfer, mice were irradiated with 500 R one or two hours before transfer.

Induction of Acute EAE in mice

All BALB/c mice were irradiated with 350 R two days before MSCH inoculation. but SJL animals were not exposed to irradiation. Lyophilized MSCH was suspended in sterile PBS at a concentration of 60 mg/ml and mixed with an equal volume of IFA supplemented with 4 mg/ml of *Mycobacterium*

tuberculosis strain H37RA and 0.5 mg/ml of *Mycobacterium butyricum*. A total of 0.1 ml of this mixture was injected into 4 foot pads of each animal. On days 1 and 3 after injection a total of $3x10^9$ *B. pertussis* bacteria in 0.1 ml were injected intravenously into each animal.

Virus Infection of Mice

Virus stock was diluted in sterile PBS and different amounts of virus were inoculated i.p. into each animal in a 0.1 ml volume 10 days before irradiation of BALB/c mice, 10 days before induction of EAE in SJL/J mice, or different days before or after sensitization with MSCH in BALB/c mice (specified in each Table or Figure).

Preparation of brain tissue for histological examination

The mice were anesthetized with ether and perfused with 5% glutaraldehyde in phosphate buffer, pH 7.4. Brains and spinal cords were carefully removed and samples were taken from brain hemispheres, pons, cerebellum, medulla and from cervical, thoracic and lumbosacral areas of the spinal cord. The samples were postfixed in 1% osmic acid, dehydrated and embedded in Epon. The 1 µm thick sections were stained with toluidine blue and thin sections were contrasted with uranium and lead salts. Electron microscopic examination of each specimen was performed by a neuropathologist without knowlege of the source of specimen.

Hematoxylin-Eosin Staining of Brain Specimen

Frozen mouse brains were cut to 5-10 µm thickness in a Cryostat at -30° C. Slides were fixed in Zenker's fixing solution for 24 h at room temperature and washed in cold running water until the water was clear. Samples were dehydrated in 80% ethanol or stored in 80% ethanol if samples were not to be stained immediately. Slides were dipped in alcohol iodine (0.5% iodine in 95% ethanol) for 1 minute to remove the mercury precipitate and rinsed in water. Samples were further stained in hematoxylin for 1-2 min and rinsed in water. Differentiation was done in 1% ammonium hydroxide solution for 3-5 seconds or until a blue color appeared and then rinsed in water. Counterstain was done with 0.5% eosin in 95% ethanol. The slides were then dehydrated rapidly in two changes of 100% ethanol. Further clearing was done in two changes of xylene, 1 min each time. Slides were mounted with Permount and examined with a light microscope.

Clinical Rating of EAE Mice

Mice were evaluated neurologically according to a 4 point scoring system (- = normal; + = fur ruffing and tail atonia; ++ = slight hind limb paresis or paralysis; +++ = hind limb paralysis; ++++ = moribund.).

Virus infectivity assay

Supernatants of infected spleen cells were collected daily for six days and virus titers were determined as $TCID_{50}$/ml on the MBA-13 cell line.

Infectious Center Assay

Single-cell suspensions of spleen and lymph node cells, free of erythrocytes, were prepared in RPMI 1640 complete medium. The mononuclear cells were washed extensively (at least three times) to remove free virus and then assayed for infectious centers by plating on a confluent monolayer of MBA-13 cells in 35 mm wells in six-well dishes (Costar, Cambrige, Massachusetts). After adsorption for 60 min at 37° C, the cells were overlaid with 3 ml of 0.5% Seakem agarose (FMC Corp., Marine Colloids, Grand Island, New York) supplemented with 5% heat-inactivated FBS, 200 IU/ml of penicillin-G, 200 µg/ml of streptomycin, and 1% glutamine. The plates were incubated for 4 days at 37° C and stained with staining solution containing 85% of crystal violet, 10% ethanol and 5% formalin. To correct for plaques that may have been caused by residual free virus remaining after 3 washes, the cell-free supernatant was also plated on MBA-13 cell monolayers. Plaques produced by this supernatant were subtracted to determine the number of infectious centers.

Infection of Macrophages by SFV

For measurement of virus production, virus at a m.o.i. of

10 was added to macrophage cultures, and adsorbed for 1 h at 37° C in an humidified CO_2 incubator. After 3x washing with PBS, residual virus on the cell surface was neutralized with a polyclonal anti-SFV serum for 1 h at 4° C, and the cells were further washed 3x before incubation at 37° C. The last wash was collected for determination of residual infectious virus. No significant difference was observed between neutralized and non-neutralized macrophages in terms of release of residual surface-associated virus. For studies of IL-1 production and Ia expression on infected macrophages, different m.o.i. of virus were used, and infected macrophages were not neutralized with anti-SFV antiserum at 4° C.

Preparation of Mouse Brain Specimens for Determination of Infectious Virus after A7-SFV Infection in vivo

Mouse brains were taken from mock infecetd or infected mice at different times after virus infection. Brains were washed 3X in PBS and weighed and homogenized in cold PBS. One ml PBS was usually added to one mouse brain. After homogenization, each sample was centrifuged at 2,000 RPM for 10 min., the supernatant was harvested and infectious virus detected with a standard microtitration assay as described above. Brains were sometimes stored at -70° C until homogenized.

Preparation of Mouse Blood Specimens for Determination of

Infectious Virus after A7-SFV Infection in vivo

Mouse blood was harvested by heart puncture from mock infected or infected mice at different times after virus infection. Blood was stored at 4° C overnight and centrifuged at 1,000 RPM for 5 min. Sera were then harvested and titrated with a standard microtitration assay as described above. Approximately 0.2 ml serum could usually be collected from each mouse.

Purification of β-propiolactone Inactivated SFV

The supernatant was harvested from MBA-1 cells infected with W-SFV by centrifugation at the time when > 3+ of CPE was observed. The virus titer was usually 10^8 PFU/ml. The virus suspension was concentrated 20-fold with a Millipore MiniTan apparatus (Millipore Corp., Massachusetts), and a sucrose step gradient used to purify the SFV (ranged 20, 25, 31.3, 37.5, 43.8, 50, 60%, W/V). The gradient was centrifuged for 18 h at 24K in a SW28 rotor in a Beckman ultracentrifuge (Model L8-80). Virus banding just above 43.8% was harvested and washed in PBS and centrifuged at 30K for 4 h. Pellets were dissolved in 500 µl of PBS and inactivated with β-propiolactone.

Fresh 1% β-propiolactone stock solution in 0.2 M $NaHCO_3$ containing 0.14 M NaCl was prepared. This solution was added to the virus suspension in a final concentration of 0.02%. The mixture was shaken at 4°C for 10 min and incubated for 2 h at 37°C. Another overnight incubation at

4°C was done to complete the hydrolysis of BPL. Infectivity tests were done on the MBA-13 cell line to test for residual virus. Virus protein was determined by standard Lowry test and purity was examined by SDS-PAGE.

Inactivation of Virus with UV Irradiation

Virus suspension was added to a petri dish to a depth of 0.2 cm and exposed to 1 h of UV irradiation from a germicidal bulb at a distance of 20 cm. The suspension was examined after each irradiation for efficiency of inactivation on MBA-13 cell line and no infectious virus was found.

Extraction of RNA from Mock Infected or Virus Infected Macrophages

SFV infected or mock infected peritoneal macrophage cultures were harvested at each time interval and washed twice in cold PBS and kept frozen in liquid nitrogen until further used. The frozen cells were thawed slowly on an ice bath and the total RNA isolated according to the procedures described by Kumar and Linberg (235) and Glickman *et al.* (236) with few modifications. Briefly, the samples were resuspended in 0.5 ml TNE (150 mM NaCl, 10 mM TrisCl, pH 7.8 and 2 mM EDTA) followed by the addition of equal volume lysis buffer (TNE containing 2% NP40). The lysis was carried out on ice for 20 - 30 min. and the viscosity of the sample was reduced by passing samples through an 18.5

gauge needle. After separating nuclei and cell debris by centrifugation the supernatant was mixed with an equal volume of urea buffer (10 mM TrisCl, pH 7.5, 10 mM EDTA, 350 mM NaCl, 7M Urea and 1% SDS). The samples were then extracted 2x with phenol:chloroform saturated with sodium acetate, NaCl and EDTA and precipitated overnight at -20° C with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The pellets were washed with 70% ethanol and RNA samples stored at -70° C until further used. In some experiments the samples were lysed in the presence of 100 U/ml RNAse inhibitor (RNasin, Amersham, Ontario) and 0.1% sodium deoxycholate followed by treatment with Proteinase K (Boehringer Mannheim, Dorval, Quebec) at a final concentration of 0.25 mg/ml (237). However there was little difference in the amounts of RNA recovered by these two methods. An average of 30-50 µg of RNA was recovered from 2-4 x 10^7 infected cells.

Extraction and Slot Blot Hybridization of Brain RNA from Mock- or Virus Infected BALB/c Mice

Mouse brains were taken at different times after mock- or virus infection. After 3x washing with PBS, brains were fast frozen in liquid nitrogen and stored at -70° C until used. Frozen brains were homogenized and lysis buffer was added. The rest of the extraction procedure was the same as described for macrophage RNA.

Detection of SFV RNA with Slot Blot Hybridization

Total RNA samples (20 µg each) were denatured in the presence of 50% formamide and 2.2 M formaldehyde at 65° C for 15 min. The sample volume was adjusted to 0.2 ml with 6x SSC (1xSSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and spotted to GeneScreenPlus hybridization membrane (New England Nuclear, Montreal, Quebec) using a Bio-Dot SF slot blot apparatus (Biorad, Richmond, Califonia). The membrane was air dried and baked for 2 h at 80° C in a convection oven. Prehybridization was carried out at 42° C for 18-20 h in a solution containing 50% deionized formamide, 5x SSPE (1xSSPE is 150 mM NaCl, 10 mM NaH2PO4 and 1mM EDTA, pH 7.4), 5x Denhardt's solution, 0.1% SDS and 0.25 mg/ml denatured salmon sperm DNA (237) with further additions of 50 µg/ml poly A RNA and 50% dextran sulfate. Hybridization was carried out at 42° C for 20-24 h in a similar solution containing 0.1% SDS and 1x Denhardt's solution.

The probe was derived from the plasmid pL1-SFV (a gift from Dr. A. Helenius, Yale University, New Haven; 238) containing a 4 Kb cDNA insert of SFV representing c-E1-E2-E3 regions. The plasmid DNA was digested with restriction enzymes BamHI and XbaI to release the 4 Kb SFV insert. Following the sequence information of SFV clones (239), the sites of restriction enzyme Sau3A1 which releases a large fragment (~0.9 - 1.0 Kb) from the E1 region and two medium size fragments (~0.6 - 0.8 Kb) from the E3 and E2/6K region

were isolated and purified following electrophoresis on low melting point agarose (BRL) gel.

The probe fragment was labelled by nick translation (Boehringer Mannheim) to a specific activity of $1.2x10^8$ cpm/μg DNA and used for hybridization. The hybridized blot was washed 4x at room temperature (15-30 min each) in 2xSSC followed by 4 washes at 65-68° C (30 min each), two each in 1 x SSC and 0.2 x SSC, respectivelly. All wash solutions contained 0.1% SDS. Washed membrane was dried with blotting paper, wrapped in Saran wrap and autoradiographed using Kodak XAR-5 film at -70° C with Cronex lightning plus intensifying screens. The experiment was repeated three times with RNA samples from different batches using probes from the E1 and E3/E2 regions. The blot was stripped of its probe by boiling method (237) and rehybridized with a new one whenever necessary.

Preparation of Splenocytes, and Their Adherent (ad+) and Nonadherent (ad-) Subpopulations

Spleens were obtained from 6 to 8 week old female BALB/c mice. The spleens were minced, cells squeezed through a sterilized steel mesh, and the red blood cells lysed by treating the cell suspension with Tris buffered-ammonium chloride (0.83%, w/v, pH 7.2). After three washings in PBS, ad+ and ad-cells were separated by two adhering cycles to a plastic surface for 1 h at 37°C. Immunofluorescence showed that 70-80% of adherent cells were positive for the

Mac-1 surface marker. All cell populations were maintained in RPMI 1640 medium (GIBCO Laboratories, Burlington, Ontario) supplemented with 2 x 10^{-3} M glutamine, 5 x 10^{-5} M 2-mercaptoethanol , 200 I.U./ml penicillin-G, 200 µg/ml streptomycin, and 10% heat inactivated FBS (complete RPMI medium). *In vitro* infection of spleen cells or different subpopulations were done with A7-SFV or W-SFV at different m. o. i. After one h incubation at 37 C in CO_2 incubator, cells were washed 3x with PBS. Supernatants were collected at different times after infection and infectious virus produced by these cells was assayed with a standard microtitration assay.

Preparation of Lymph Node and Peritoneal Cells

A single lymph node cell suspension was made from the draining inguinal and axillary lymph nodes of BALB/c mice treated with different regimens according to individual experiments. Peritoneal exudate cells (PEC) were harvested by peritoneal lavage using 5 ml PBS supplemented with 10 units heparin/ml. Contaminating red blood cells were lysed with Tris buffered ammonium chloride solution as above. All cells were maintained in complete RPMI medium unless otherwise specified in individual Tables or Figures.

Preparation of Thioglycollate-Elicited Peritoneal Macrophages (PM)

Cells were obtained from 6-8 wk old BALB/c mice as

described by Stewart *et al.* (216). Briefly, mice were injected intraperitoneally with 2 ml Brewer's thioglycollate broth medium (Difco Laboratories, Detroit, Mich.) 72 h before harvest. Cells were collected by flushing the peritoneal cavity with phosphate buffered saline, pH 7.0, and washed 3X with PBS before seeding onto 12 well plastic plates (Linbro, Flow Laboratories, Mississauga, Ontario). Red blood cells were lysed with Tris buffered ammonium chloride, pH 7.2. Nonadherent cells were removed by 3x washing with PBS after 2 h incubation on a plastic surface at 37° C in a humidified 5% CO_2 atmosphere. The adherent cell population had 90-93% Mac-2 positive cells as determined by flow cytometry (FACS) with specific antibodies (Hybritech, San Diego, CA.). Macrophages were maintained in RPMI 1640 complete medium. Macrophages incubated for 48 h were used as resting macrophages.

Preparation of Bone Marrow Derived Macrophages (BMDM)

Preparation of bone marrow derived macrophages have been described previously (169, 170). Briefly, cells were obtained from BALB/c mice by flushing out bone marrow with medium, and cells grown at 37° C in Corning 100x20 mm plastic dishes. Each dish was seeded with $8x10^5$ cells in 25 ml of Dulbecco's modified minimal essential medium (DMEM) containing 10% FCS, 10% horse serum, 200 units/ml penicillin-G, 200 µg/ml streptomycin, and 10% L-cell-conditioned medium (LCM) as the source of colony

stimulation factor. The cells grew exponentially to adherent colonies of pure macrophages reaching stationary phase by day 7. The cells were harvested during the logarithmic phase on day 5 with PBS containing 0.02% disodium EDTA used to dislodge the cells from the dishes.

Fractionation of macrophages by size was performed by the velocity sedimentation in FBS (169, 170). Usually 75-85% of cells were recovered after fractionation to 40 fractions which were pooled arbitrarily into four large pools (A to D) for experiments. All cells were maintained in complete Dulbecco's minimal essential medium at 37° C in a humidified incubator with 5% CO_2 atmosphere.

Immunofluorescence

For surface antigen detection, cells were suspended in a predetermined optimal dilution of the primary antibody (rat anti-Mac-1; Hybritech, Inc., San Diego, California) in a volume of 0.2 ml and incubated for 30 min in an ice bath. After three washings with PBS supplemented with 5% FBS, fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG (Cooper Biomedical, Inc., Malvern, Pennsylvania) was added in a volume of 0.2 ml. After further incubation for 30 min in an ice bath and two PBS wash cycles, cells were suspended in buffered glycerol (PBS:glycerol, 1:1 v/v). A drop of the cell suspension was transferred to a microscope slide and examined with an incident light fluorescence microscope (Leitz, Wetzlar,

FRG).

For fixed preparations, cells were centrifuged onto glass slides (Shandon Cytospin, John's Scientific, Toronto, Ontario) and fixed in cold acetone for 15 minutes at -20° C. Primary antibody (rabbit anti-SFV antibody, a gift of Dr. Sirkka Keränen, University of Helsinki, Finland) was added and the slides incubated for 30 min at 37°C in a humidified chamber. After three washings with PBS, rhodamine-conjugated goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland) was added and incubated as above. Following three washings in PBS, a drop of buffered glycerol was added to each slide, examined with a fluorescence microscope. Normal rat or rabbit serum was included in each experiment as a nonspecific control.

The simultaneous presence of the surface marker Mac-1 and viral antigen in different cell population of spleen cells was determined by combining the above procedures. After staining for surface immunofluorescence, cells were centrifuged onto glass slides and fixed in acetone for 15 min at -20° C. Staining for SFV antigens was then done as described above.

For surface antigen detection on macrophages, monolayers of cells in chamber slides (Lab-Tek Products, Division of Miles Laboratory, Inc., Illinois) were stained with a predetermined optimal dilution of the primary antibody (1 µg/ml; rat anti-Mac-2; Hybritech, Inc., San

Diego, Colifonia) in a volume of 0.2 ml and incubated for 30 min in an ice bath. After 3x washing in PBS supplemented with 5% FBS, fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Cooper Biomedical, Inc., Malvern, Pennsylvania) was added in a volume of 0.2 ml. After further incubation for 30 min in an ice bath and 2x PBS washes, cells were overlaid with a drop of buffered glycerol (PBS:glycerol 1:1, v/v) and cover slips and examined with a fluorescence microscope. Normal rat serum was included in each experiment as a nonspecific control.

Analysis of Surface Antigens of Different Cell Populations by Flow Cytometry (FACS)

Lymph node and spleen cells from mice treated by different regimens were stained with anti-Ia ($H\text{-}2^d$) or anti-Mac-1 monoclonal antibodies (rat anti-mouse Ia; rat anti-mouse Mac-1; Hybritech), or anti-L3T4 (GK 1.5, ATCC), or anti-Lyt. 2 (53.6-72, ATCC) prepared in this laboratory, and subsequently fluorescein-conjugated goat anti-rat IgG (Cooper Biochemical). All staining were done at 4° C. Normal rat IgG was used as a nonspecific control in each experiment. One million 1% formaldehyde fixed cells were analysed by FACS (MDADS, Coulter Electronics, Inc., Hialeah, Florida) at an excitation wavelength of 488 nm and the results expressed as cell number/log scale of fluorescence intensity. One or two parameter analysis were used. The results were presented as positive cell number

(percentage). In determination of Ia antigen on PM, PM was removed from plastic plates by incubation of cells with 2 mM EDTA solution at room temperature for 15 min. The staining procedure was the same as above and the results were also expressed as Induction Index (I.I.):
% positive cells (i. i.) = (% virus infected cells - % noninfected cells)/ % noninfected cells

Lymphocyte Transformation Assay

Lymph node or spleen cells were washed 3x in PBS after removing contaminated red blood cells with Tris-NH_4Cl as above and dispensed at a concentration of 2 x 10^5 cells/0.1 ml/well in a 96-well round bottom plate (Linbro, Flow Laboratories, Inc., Mississauga, Ontario). A predetermined optimal concentration (2.5 µg/ml) of concanavalin A (ConA, Sigma, St. Louis, Missouri) or guinea pig myelin basic protein (10 µg/ml), or proteolipid protein (PLP, 6 µg/ml) or purified, inactivated SFV (6 µg/ml) or ovalbumin (OVA, Sigma, St. Louis, Missouri) in 0.1 ml of medium or control medium was added and the plates were incubated at 37° C in a 5% CO_2 humidified atmosphere. The cell cultures were pulsed with 0.4 µCi [^{3}H]-methylthymidine for the last 18 h of the 48 h incubation period and harvested with an automated cell harvester (Titertek 550, Flow Laboratories, Mississauga, Ontario) on a glass fibre filter. The amount of incorporated radioactivity was measured by scintillation spectrometry. Cultures were done in quadruplicate and

results expressed as the mean ± standard deviation in each experiment or mean ± standard deviation of three or four separate experiments or as a stimulation index (S.I. = cpm of cells with antigen stimulation/cpm without antigen stimulation).

Trypan Blue Exclusion Test

Seventy microliters of trypan blue dye (Sigma; fresh solution: 4 volumes of 1% trypan blue plus one volume of 4.25% NaCl) were added to $3x10^5$ cells in 300 µl and the percentage of dead cells was calculated.

Activation of Macrophages with Rat ConA Supernatant

Macrophages were activated *in vitro* by the addition of a lymphokine preparation consisting of the culture supernatant fluid of Con A activated rat spleen cells (rat ConA sup). The rat ConA sup was prepared as following: rat spleen cells were incubated in RPMI 1640 complete medium containing an optimal concentration of ConA (2.5 µg/ml). The supernatant was harvested after 48 h incubation at 37°C. To remove residual Con A Sephadex G-100 beads were added to the supernatant (10% w/v), and the mixture was kept at 4°C with vigorous stirring overnight. After removal of the beads by centrifugation, the supernatant was passed through a filter paper (Whatman Limited, England) and stored at -70° C until used. The optimal concentration of this supernatant for activation of macrophages was

determined to be 10% (v/v).

Preparation of IL-1, IL-2 and IFN Containing Supernatants from Cells Prepared from Mice Mock- or SFV-infected in vivo

Supernatants for IL-1 and IL-2 assays were prepared according to Gearing *et al.* (191). Briefly, for IL-1 assay, cells of different population from mice treated with different regimens were incubated in complete RPMI medium containing 2.5 μg/ml ConA. After 24 h incubation, the cultures were centrifuged at 1,000xg for 10 min. The supernatants were collected, UV-irradiated for 1 h and stored at -70°C. The supernatants for IL-2 and IFN assays were prepared in a similar manner but were incubated for 48 h. The supernatants were tested for infectivity on the MBA-13 cell line after UV irradiation and were found to be noninfectious. Residual ConA was neutralized by adding β-methylmannoside (final concentration, 1 g/50 ml of culture supernatant).

Preparation of Interleukin-1 Containing Supernatant from Thioglycollate Elicited Peritoneal Macrophage Cultures

In each experiment, macrophage cultures were divided into different groups: Group 1, mock or infected with virus and without stimulation; Group 2, mock or infected with virus and stimulated with a predetermined optimal dose of LPS (10 μg/ml; Sigma, St.Louis, MO.); Group 3, mock or infected with virus and stimulated with recombinant IFN-γ (100

I.U./ml; American Cancer Society Inc., a kind gift of Dr. Chester Stock). The supernatant was harvested at 4, 8, 16, 20, 24 and 48 h p. i.. U.V. inactivation was done on supernatants before the IL-1 assay, and no residual infectious virus was detected.

Indomethacin Treatment of SFV-infected and Mock-Infected Thioglycollate Elicited Peritoneal Macrophages

Macrophages treated with different regimens were incubated in RPMI complete medium containing 1 µg indomethacin (Sigma, St. Louis, Missouri) for the full incubation period at 37° C. The supernatants were harvested and stored at -70° C. Control cultures consisted of uninfected macrophages treated with indomethacin and infected macrophages without indomethacin.

Interleukin-1 assay

One hundred microliters of each supernatant was added to an equal volume ($2x10^5$ cells/well) of murine thymocyte suspension in a 96-well round bottom plate (Linbro) and the culture stimulated by a suboptimal concentration of ConA (1.25 µg/ml). The capacity of samples to support proliferation of thymocytes was measured by uptake of [^{3}H]-methylthymidine during the last 18 h of the 48 h incubation period. The results were expressed as the mean ± standard deviation of the incorporated [^{3}H]-thymidine in triplicate or quadruplicate cultures.

Interleukin-2 assay

A total of $2x10^4$ IL-2 dependent CTL line cells per well in a volume of 100 µl was seeded into a 96-well round bottom plate. Samples of test supernatants (0.1 ml) were added. The ability of the supernatants to support proliferation of CTL line cells was measured by [^{3}H]-methylthymidine uptake during the last 18 h of the 48 h incubation time. Control wells consisted of cells in medium with or without ConA and cells in a standard supernatant with a known amount of IL-2 described as above. The results were expressed as the mean ± standard deviation of the incorporated [^{3}H]-thymidine in quadruplicate cultures or calculated as units/ml using a recombinant IL-2 standards (Genenzyme, Boston, Massachutts).

Interferon(IFN) Assay

IFN was assayed by testing the ability of a virus free supernatant to protect monolayers of L929 cells from the cytopathic effect (CPE) of vesicular stomatitis virus (VSV). Briefly, two-fold serial dilutions of test samples, in duplicate, were made in Eagle's MEM (EMEM; 0.1 ml per well) supplemented with 4% FBS in a 96 well plate. Reference samples of IFNα,β and IFNγ were tested in parallel, and the culture medium served as a negative control. Plates containing the samples were incubated with 5×10^4 L929 cells/0.1 ml of EMEM supplemented with 4% FCS

for 24 h at 37°C in a 5% CO_2 atmosphere. At confluence, cells were inoculated with 10 m.o.i. of VSV in 100 ml of EMEM containing 2% FBS. Cultures were incubated until control cultures showed 100% CPE. The medium was aspirated from each well and the monolayers were stained with a solution of PBS containing 5% crystal violet, 10% ethanol (95%), 1% formaldehyde for at least 30 min, washed in tap water, and air dried. The IFN titer was read as the reciprocal of the dilution that protected 50% of the cell monolayer from cytopathic effect. Values were compared to the IFN standards and expressed as units per ml of fluid.

Antigen Presenting Assay

Lymph node cells prepared from OVA immunized mice (see above) were incubated with RPMI complete medium with 50 µg/ml of OVA for 96 h at 37° C at a 5% CO_2 atmosphere. After incubation, dead cells were removed by Ficoll-Paque™ centrifugation and live cells were collected and washed 3x in PBS containing 5% FCS. These cells contained >99% Thy.1 positive cells according to FACS analysis and were used as OVA specific T cells in antigen presenting assays.

Spleen macrophages were used in this assay: Spleen cells obtained from mice mock-infected or infected with A7-SFV *in vivo* at different times were irradiated with 1500 R and used as a source of spleen macrophages. Cells irradiated with 1500 R did not proliferate in response to mitogen stimulation.

The ability of the macrophages to present antigen to T cells was measured by the uptake of [^{3}H]-methylthymidine by T cells. In each experiment, different numbers of T cells were added to varied numbers of macrophages in the presence of 50 µg/ml of ovalbumin (final concentration). Control groups included: T cells and macrophages; T cells and antigen only; T cells only. Cultures were pulsed with 0.4 µci of [^{3}H]-methylthymidine for the last 18 h of a 96 h incubation time. Results were expressed as mean ± standard deviation of quadruplicate cultures in each experiment.

Purification of MBP and PLP

MBP was purified from guinea pig spinal cord (53, 54) and was a kind gift of Dr. D. L. Tyrrell of this department. Partially purified proteolipid protein (PLP) was obtained from Dr. V. K. Tuohy (Biochemistry Department, E. K. Shriver Center, Harvard Medical School) and the purification procedure has been described elsewhere (188, 189, 190). Conversion of PLP to an aqueous phase was done as follows: An amount of 3 - 4 ml of PLP in chloroform : methanol : acetic acid was placed into a clean, dry glass dish deep enough to swirl the dish without splashing the liquid. During this entire process, a stream of nitrogen gas was blown softly over the fluid to prevent oxidation of PLP when it entered the aqueous phase. One drop of double distilled deionized water was added and fluid was stirred by swirling dish to prevent aggregation of precipitate

entering the aqueous phase. Swirling was continued until the fluid had no cloudiness in it. More water was added and the fluid was allowed to stay in the nitrogen stream for 15-20 min to evaporate any remaining methanol. Deionized distilled water was used to replenish the approximate original volume. To remove acetic acid, solution was dialyzed overnight in large bath of double distilled deionized water. Standard dialyzing tubing with a m. w. cutoff of 12,000 - 14,000 (Fisher Scientific, Pittsburgh, PA 15219) was used. A Lowry test was used to measure the protein concentration and the PLP solution was stored at -70° C until used.

In vivo Administration of anti-L3T4 or anti-Lyt.2 or F4/80 Monoclonal Antibodies in Mice

One mg of MAb to L3T4 or Lyt.2 or F4/80 in a volume of 0.3 ml was administrated by tail vein into each mouse treated with different regimens at day 5 after neuroantigen inoculation (2 days before virus infection). The optimal dose which can completely inhibit the allogenic mixed lymphocyte reaction (allo-MLR) and/or anti-H-2^b CTL response was determined by Dr. M. Sadalin (Department of Immunology, University of Alberta). It was demonstrated that 5 days after *in vivo* administration of anti-L3T4 MAb or anti-Lyt 2.2 MAb with doses used in this work, the allogenic MLR or anti-H-2^b CTL responses were completely abolished respectively. Normal rat IgG was injected as

nonspecific control. Clinical observation were done as described above.

In vivo Administration of Recombinant IFNγ in Mice Treated with Neuroantigen

Groups of mice were irradiated with 350 R, MSCH and pertussis vaccine was administered according to a standard regimen described above. Instead of injecting A7-SFV at day 7 after MSCH inoculation, 10,000 units of recombinant IFNγ per mouse was inoculated into the tail vein of mouse at day 9 after MSCH inoculation. The same dose was repeated at days 10 and 11. Control mice received PBS or were infected with virus at day 7 after MSCH inoculation. Clinical observations were done as described above.

Induction of EAE in Naive BALB/c Mice with Passive Cell Transfer

Before passive transfer, spleen or lymph node cells were incubated with ConA (2.5 µg/ml) or MBP (25 µg/ml) for 96 h *in vitro*. After incubation cells were collected and dead cells were removed with Ficoll-Hypaque™ centrifugation at 1200 RPM for 25 min (Beckman, Model TJ-6). Live cells were harvested and washed 3x in PBS containing 5% FCS. Different numbers of cells in a volume of 0.2 ml RPMI medium with 5% FCS were injected into the tail veins of recipient mice irradiated 1 - 2 h before inoculation. On days one and three p. i. $3x10^9$ *B. pertussis* bacteria suspended in 0.1 ml

of PBS were injected into the tail vein.

Transfer of A7 Primed Lymphocytes to Mice Induced to Develop EAE

Groups of mice were treated with 350 R, MSCH and pertussis vaccine according to the standard regimen described above. Instead of injecting A7-SFV at day 7 after MSCH inoculation, $5x10^7$ spleen cells prepared from BALB/c mice infected with A7-SFV (10^6 PFU/mouse, i.p.) 5 days before preparation were inoculated into each mouse by the tail vein route. Control groups included: a. Mice with MSCH inoculation and PBS at day 7; b. Mice with MSCH and normal spleen cells; c. Mice with MSCH and virus at day 7; d. Mice with virus at day 7 only; e. Mice with spleen cells from A7 infected mice only. Spleen cells were also examined for infectious virus with an infectious center assay and no infectious virus was found. Clinical observations were done as described above.

In a separate experiment, different fractions of spleen cells from A7-SFV infected mice were injected at day 7 after MSCH inoculation which included the following groups: a.Non- fractionated A7-SFV primed spleen cells; b. A7-SFV primed spleen cells depleted of B cells by passing through a T cell enrichment column; c. A7-SFV primed spleen cells depleted of ad+ cells by two cycles of one h adhering to plastic plates; d. A7-SFV primed spleen cells irradiated with 1500 R; e. Normal spleen cells. The cell numbers used

are shown in the footnote of the Table.

Induction of EAE in Naive Mice with Mixed Cells from Mice Treated with Different Regimens

A group mice was treated with 350 R and MSCH and pertussis vaccine according to the standard regimen (group A). Another group of mice was infected with A7 (10^6 PFU/mouse i.p.) 5 days before preparing cells (group B). Lymph node or spleen cells obtained from group A were incubated with ConA (2.5 μg/ml) for 96 h and dead cells were removed through Ficoll-Paque™ centrifugation (A Cell). Fresh lymph node or spleen cells were prepared from group B (B Cell). $2x10^7$ A cells were mixed with $2x10^7$ B cells in a total volume of 0.4 ml (Cell A+B). Cell A+B was injected into each mouse received 500 R 2 h before transfer by the tail vein. Pertussis vaccine was inoculated at day 1 and day 3 after transfer. Clinical observation were done as described above.

B Cell Depletion by T Cell Enrichment Column

T cell columns were purchased from SCICAN (Edmonton, Alberta, Canada) and stored as instructed. Columns were washed with a minimum of 15 ml balanced salt solution (BSS). While the column was washing, thawed antisera (goat anti mouse Ig, SCICAN) was diluted with BSS. Just before the 15 ml of BSS reached the top level of the column bed, the entire contents of one vial of antiserum was added to

each of the column to be used. When the liquid level of the antisera reached the top of the column bed, the flow of the column was stopped and the column incubated for about 2 h at room temperature. After that, the column was further washed with 20 ml of BSS. During this wash, the flow rate was adjusted to 6 - 10 drops per min. When the last of the 20 ml of the wash reached the top of the column bed, $1.5x10^8$ spleen cells in the volume of 1.5 ml was added to the column. When the sample reached the top of the column bed, more BSS was added and the eluting drops were collected. At least 15 ml efferent fluid was collected and washed and used for transfer experiment.

Effect of A7-SFV Infection in vivo on OVA Induced Cellular Immunity

10^6 PFU of A7 virus was inoculated into each mouse i.p. day 7 after OVA immunization. The OVA immunization protocol was as follows: 200 µg/ml chicken ovalbumin in PBS (OVA; Sigma) was homogenated with equal amount of CFA (Difco. Lab., Michigan). 200 µl of the mixture was injected into 4 footpads of each mouse. Control groups included: a. Mice immunized with OVA only; b. Mice infected with virus only. At different days after OVA immunization and virus infection, lymph node cells were prepared as above. Mitogen or antigen driven proliferation was done as described above.

Statistics

Statistical significance of differences between experimental groups was tested by the chi-square test or the Fisher exact probability test.

RESULTS

Chapter One

Induction of EAE in Genetically Resistant BALB/c Mice with Aid of Low Dose Irradiation

Effect of Whole Body Irradiation on Induction of EAE in BALB/c Mice

Low dose whole body irradiation is known to suppress the immune system, and render genetic resistant mice strains susceptible to EAE induction (12, 19, 20). Different irradiation doses ranging from 350 R to 650 R were administered to BALB/c mice two days before inoculation of neuroantigen - mouse spinal cord homogenate. All mice had slight fur ruffing after irradiation, and recovered two days later when the neuroantigen was inoculated. All mice treated with 550 R or 650 R died shortly after neuroantigen inoculation (between 3 - 7 days) (Table 1). An irradiation dose of 450 R did not render mice susceptible to EAE induction or cause death. In groups of mice treated with 350 R of whole body irradiation, about 20 - 30% of the mice developed EAE. None of the mice in the groups treated only with neuroantigen and pertussis vaccine but without treatment of low dose irradiation developed EAE until

Table 1: Effect of Total Body Irradiation on EAE Induction In BALB/c Mice[a]

Irradiation	Mice with EAE[b]	Dead/Total
None	0/30	0/30
350 R	8/30	0/30
450 R	0/30	0/30
550 R	0/30	30/30

a: The mice were inoculated with MSCH in adjuvant and pertussis bacteria were injected on day 1 and 3 after inoculation of MSCH. The clinical signs of EAE were observed daily for 30 days.
b: None of the animals developed clinical EAE in the groups injected only with MSCH and immunological adjuvant.

terminated by day 112 after neuroantigen inoculation.

Development of Neurologic Signs in BALB/c Mice with EAE

The earliest observable neurological signs in mice with EAE were frequently associated with fur ruffing, tail atonia (rated as +). In some mice, the initial neurologic sign was unilateral hind limb paresis or bilateral hind limb paresis (2+). Development from fur ruffing to paresis or paralysis (3+) usually took 24 h, but in some mice, it may take three days. Subsequently, about 50% of the mice with neurologic signs became moribund (4+), and were terminated at this stage. The other fifty percent of the mice with neurologic signs remained at the stage of hind limb paresis or paralysis during the entire observation period (about 40 days), and were terminated by the end of experiments. Fig. 1.1 and 1.2 showed mice with typical neurological signs of EAE - hind limb paralysis. When mice with paralysis had difficulties in reaching food or water, they were hand fed daily with water, and water soaked food pellets were kept close to the mice. Therefore, death was not likely caused by thirst or starvation. Some mice also recovered from fur ruffing and were not scored as EAE.

Characteristics of EAE in SJL/J and BALB/c Mice

SJL/J mouse strain ($H\text{-}2^s$) is known to be genetically susceptible to induction of EAE (1) and was therefore chosen as a positive control in establishing BALB/c ($H\text{-}2^d$)

Figure 1.1 (top) A BALB/c mouse with tail atonia and typical hind limb paralysis after 350 R irradiation and neuroantigen inoculation. *1.2.* (bottom) Two SJL/J mice - one with tail atonia and typical hind limb paralysis after neuroantigen inoculation (the mouse on bottom) and one without disease (the mouse on top).

mouse model. Neurologic signs of EAE in SJL/J mice were usually observed about 11 to 15 days after neuroantigen inoculation and the neurologic signs were the same as described above for the BALB/c mouse model. About 80% of SJL/J mice developed EAE after neuroantigen inoculation and pertussis vaccine injection. In contrast to the SJL/J mouse model, only about 20 to 30% mice developed EAE in BALB/c mouse model. Furthermore, the time of disease onset was much later in the BALB/c mouse model, approximately 24 days after neuroantigen inoculation (Table 2).

The following diagram shows schematically the difference between SJL/J and BALB/c mice in the induction of EAE (350 R=irradiation; MSCH= mouse spinal cord homogenate inoculation; P.V.= Intravenous injection of Pertussis bacteria; EAE=time of EAE onset - percentage of animals developing EAE is given in the brackets).

BALB/c mice:

day -2 day 0 day +1 day +3 day +24

350 R --> MSCH -----> P.V. -----> P.V. -----> EAE

(20 - 30%)

SJL/J mice:

day 0 day +1 day +3 day +12

MSCH -----> P.V. -----> P.V.-----> EAE

(80 - 100%)

Table 2: EAE Induction in SJL/J and BALB/c Mouse Strains

Mouse Strain	EAE Induction Regimens	Mice with EAE/ Total Mice	(%)	Disease Onset days P.M[d]	Clinical Assesment
SJL/J	MSCH[a] + P.V.[b]	24/30	80	13-15	+ to ++++
BALB/c	IR[c] + MSCH + P.V.	8/30	27	22-26	+ to ++++

a: Mouse spinal cord homogenate.
b. Pertussis vaccine.
c. irradiation with 350 R.
d: post MSCH inoculation.

Histological Observations of EAE in BALB/c Mice

In H & E staining, brain sections from BALB/c mice with neurological signs of EAE demonstrated perivascular infiltrates of inflammatory cells, which occasionally invaded the white matter parenchyma. Large inflammatory cuffs in the brain hemispheres and medullar area were frequently seen (Fig. 2.1). Meningitis was also a characteristic feature. Inflammatory cells were not observed in brain sections from neuroantigen inoculated, non-irradiated BALB/c mice (Fig.2.2). The perivascular infiltration of inflammatory cells (cuff) was also found in SJL/J mice with neurologic signs of EAE.

In brain sections stained with toluidine blue, focal areas of white matter destruction with perivascular inflammatory cells were observed by light microscopic examination (data not shown)

Some inflammatory cells were also observed in the subarachnoidal space. These abnormal histological findings were most often observed in the lumbar part of the spinal cord but small focal lesions were also present in the upper part of the spinal cord and, occasionally, in the cerebellum, brain stem and cerebral hemispheres. No such changes were found in non-irradiated mice treated with neuroantigen but without irradiation.

An active process of demyelination corresponding to the focal white matter changes seen by light microscope

Figure 2.1. A hematoxylin & eosin staining of brain section from BALB/c mouse with neurologic signs of EAE (hind limb paralysis). Large inflammatory cuffs in the medullan area were demonstrated.

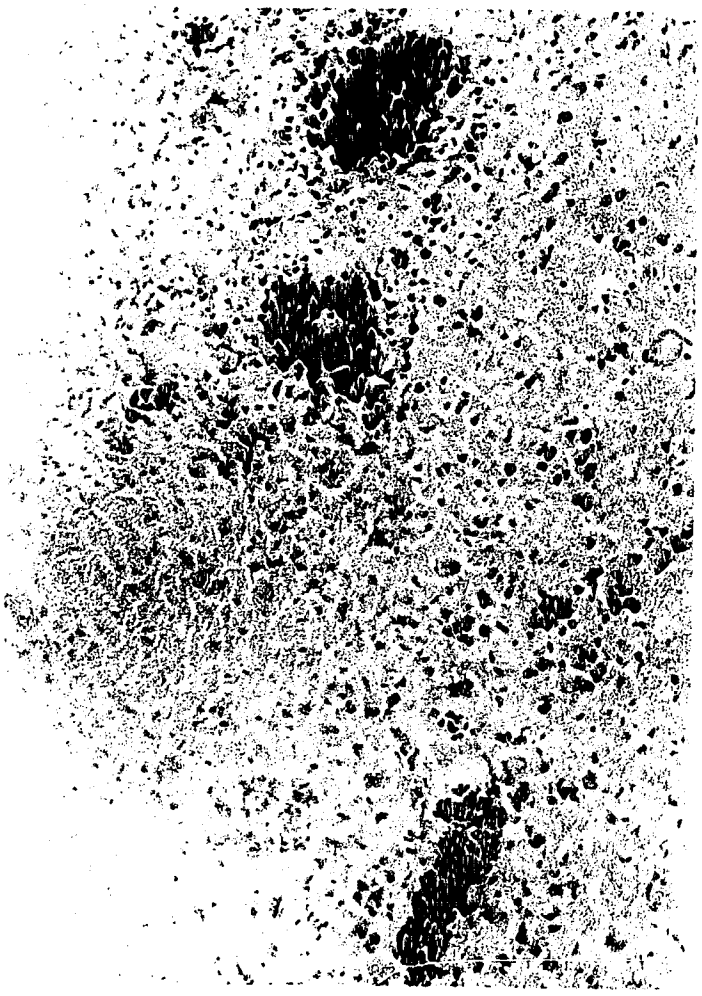
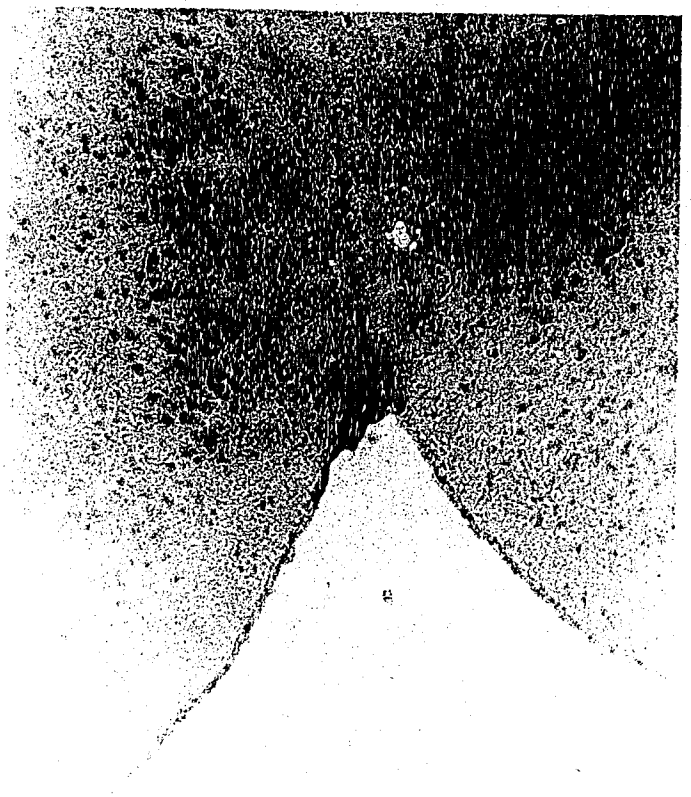


Figure 2.2. A hematoxylin & eosin staining of a brain section from a BALB/c mouse treated with neuroantigen but not irradiated. No inflammatory cuff was seen.



could be observed by electron microscopic examination. Many macrophages with intracytoplasmic phagocytosed material in subpial areas were seen. Axons which were being stripped off by macropahges were conspicuous in brain sections from BALB/c mice with 2 + neurologic signs (hind limb paralysis). In addition to macrophages, lymphocytes and some polymorphonuclear leukocytes were observed both in areas of demyelination and in the subarachnoidal space. Therefore, the pattern of demyelination observed in BALB/c mouse model agreed with the most commonly described pattern of myelin breakdown and stripping off myelin by invaded macrophages in SJL/J mouse model (1, 2).

Chapter Two

Potentiation of the Induction and Development of EAE in BALB/c Mice by SFV Infection - Basic Characteristics of the Model

Potentiation of EAE by SFV Infection at Day 7 after Neuroantigen Inoculation in BALB/c mice

A low disease incidence and late disease onset in the BALB/c model may be advantageous for observing a modulating effect of virus infection. A total of 10^6 PFU of a nonlethal strain of SFV (A7-SFV) was injected intraperitoneally into each BALB/c mouse at day 7 post neuroantigen inoculation. Intraperitoneal inoculation has the advantage of not directly disturbing the blood brain barrier by inoculation and the resulting CNS infection is more natural (160).

In the group of mice treated with 350 R, neuroantigen inoculation and virus infection, about 60% of the mice developed neurologic signs of EAE (Table 3). Slight hind limb paralysis and fur ruffing were always the initial signs of the disease onset. The earliest disease onset time was on the 12th day, and some mice had initial neurological signs on the 16th day as well. Most of the mice with

Table 3: Effect of A7-SFV Infection 7 Days after Sensitization with MSCH and Immunologic Adjuvant on the Induction of EAE in BALB/c Mice[a] The mice were irradiated with 350 R two days before induction of EAE as described in the legend of Table 1. Pertussis vaccine inoculation at day 1 and day 3 after MSCH inoculation was done as described in Materials and Methods.

EAE induction regimen	Mice with EAE/ Total no of mice	(%)	Disease onset	Clinical Assessment
IR[b]+MSCH[c]+V[d]	38/65	58.5[g]	12-16[e]	+++ to ++++
IR+MSCH	9/55	16.4	21-26[e]	+ to +++
IR+V	6/50	12.0	8-9[f]	+ to ++
V	1/55	1.8	9[f]	+
MSCH+V	0/20	0.0	-	-

a. Data pooled from 4 separate experiments.
b. Irradiated with 350R.
c. Inoculated with mouse spinal cord homogenate.
d. Infected with SFV.
e. Days after MSCH inoculation.
f. Days after virus infection.
g. Highly significantly different from other groups ($p<0.001$).

neurologic signs reached a moribound stage about 2 - 3 days after the initial signs. Some mice recovered, while some remained paralytic during the entire observation period (Table 3). In the group of mice irradiated with 350 R and inoculated with virus only, less than 10% mice died after inoculation of the virus, and a few mice developed paresis at about 9 days post virus infection. All mice showed slight fur ruffing about 2 days after virus infection and recovered afterwards. In the group of mice infected with A7-SFV, only one out of a total 55 mice died. Therefore, low dose whole body irradiation may be responsible for the increased death in mice treated with both 350 R and virus infection. Irradiation (350 R) was a prerequisite to the potentiation effect of A7-SFV on EAE, as in the group of mice inoculated with neuroantigen and infected with virus, neither EAE nor death was seen (Table 3).

Taken together, these experiments show that SFV infection or neuroantigen inoculation given alone will induce neurological symptoms in only a small number of irradiated BALB/c mice but virus infection given 7 days after sensitization with neuroantigen has a synergistic effect on the development of neurological signs of EAE.

Histological Observations

BALB/c mice from different control and experimental groups were studied for histological changes in the CNS 14 to 16 days after EAE induction.

Animals with neuroantigen inoculation and SFV infection at an early stage of the clinical disease (2+) had focal areas of white matter destruction with perivascular inflammatory cells (Fig. 3.1 - 3.3). No such changes were found at days 14 to 16 in animals with neuroantigen alone. Some animals with SFV infection had occasional perivascular inflammatory cells but no signs of brain destruction were seen (Fig. 3.4, 3.5).

Electron microscopic examination of the samples from animals sensitized with neuroantigen and infected 7 days afterwards with SFV revealed an active process of demyelination (Fig. 3.6 - 3.9) similar as demyelination observed in SJL/J or BALB/c mice with neurologic signs of EAE. The brain samples taken simultaneously from animals treated only with neuroantigen or SFV showed some oedema and slight degenerative changes but no signs of active demyelination.

Effect of Timing of A7-SFV Infection on EAE Development

SFV was inoculated i.p. at different times post neuroantigen inoculation (Table 4). Virus infection at day 7 post neuroantigen inoculation had a maximal effect, about 70 - 80% mice developed typical neurological signs of EAE. Virus infection at day 6 or 8 also rendered 60% of mice developed EAE. However, virus infection at day 3 post neuroantigen inoculation had no potentiation effect. In the group of mice treated with neuroantigen only (mock-infected

Figure 3.1 Three areas of white matter destruction (arrows) in the lateral column of the lumbar spinal cord of an animal inoculated with MSCH and subsequently infected with SFV (X55). The sample is from a mouse with an early clinical disease (++) 14 days after MSCH inoculation. The lowermost area of white matter destruction is located beside the subarachnoid vessel (asterisk) and this area is shown in higher magnification in Fig. 3.2.

Figure 3.2 A higher magnification from Fig. 3.1 showing perivascular inflammatory cells (thin arrows) beside the subarachnoid vessel (asterisk). Macrophages with intracytoplasmic phagocytosed material (arrow) can be seen in the white matter (X400).

Figure 3.3 In addition to perivascular inflammatory cells (thin arrow), a demyelinated axon (thick arrow) is seen. On the left, myelin destruction is evident (X400).

Figure 3.4 and 3.5 Mouse lumbar white matter (X380) from animals inoculated with MSCH (Fig. 3.5) or SFV (Fig. 3.6). Both samples present well-preserved myelin. Some edema but no obvious white matter destruction, macrophage or inflammatory cells around the vessels (asterisks) are present.

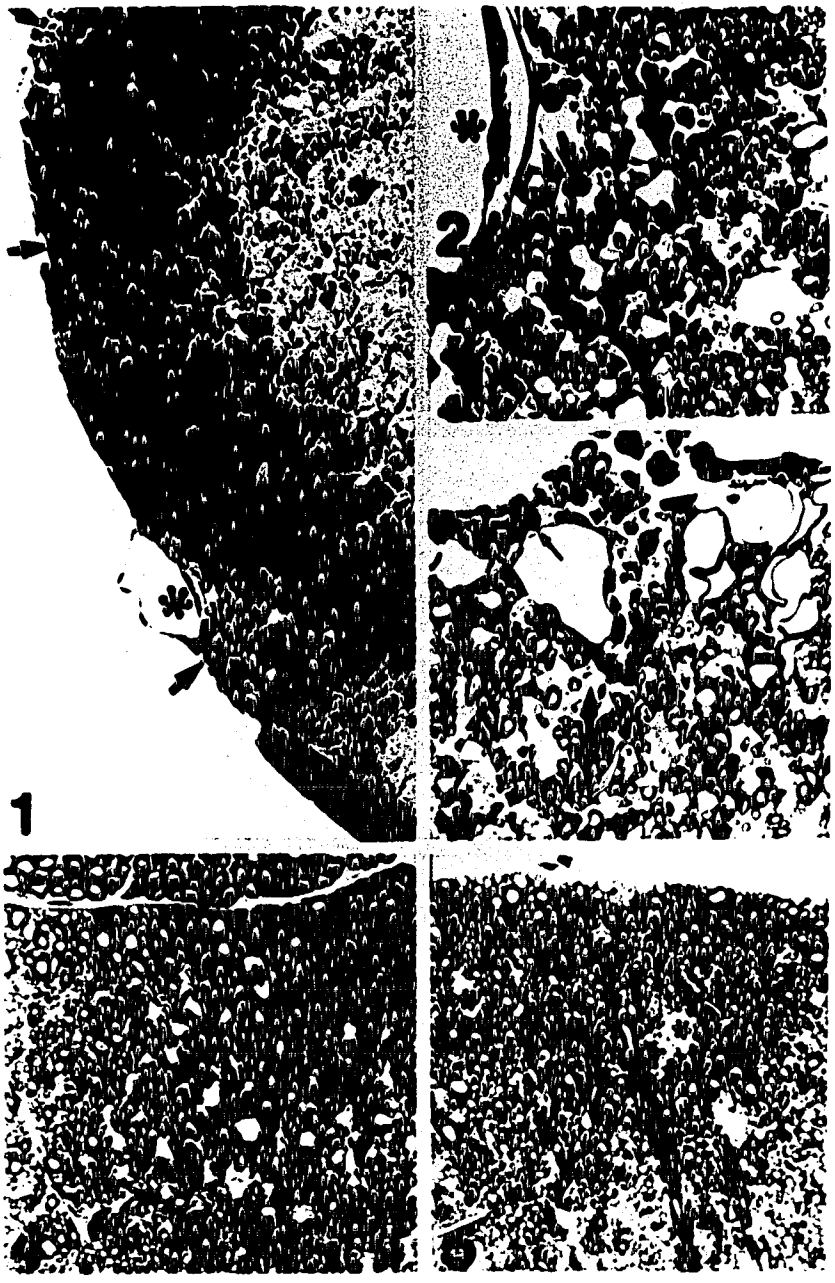

1
2

Figure 3.6 An electron micrograph from the lumbar spinal cord of a mouse treated with spinal cord homogenate and SFV. A macrophage (m) containing intracytoplasmic phagocytozed material and a polymorphonuclear leukocyte is located at the top. Beside the macrophage, two naked axons (a) and an axon in process of demyelination (arrow) can be seen (X4000).

Figure 3.7 Within areas of ongoing demyelination several demyelinated axons (X8300).

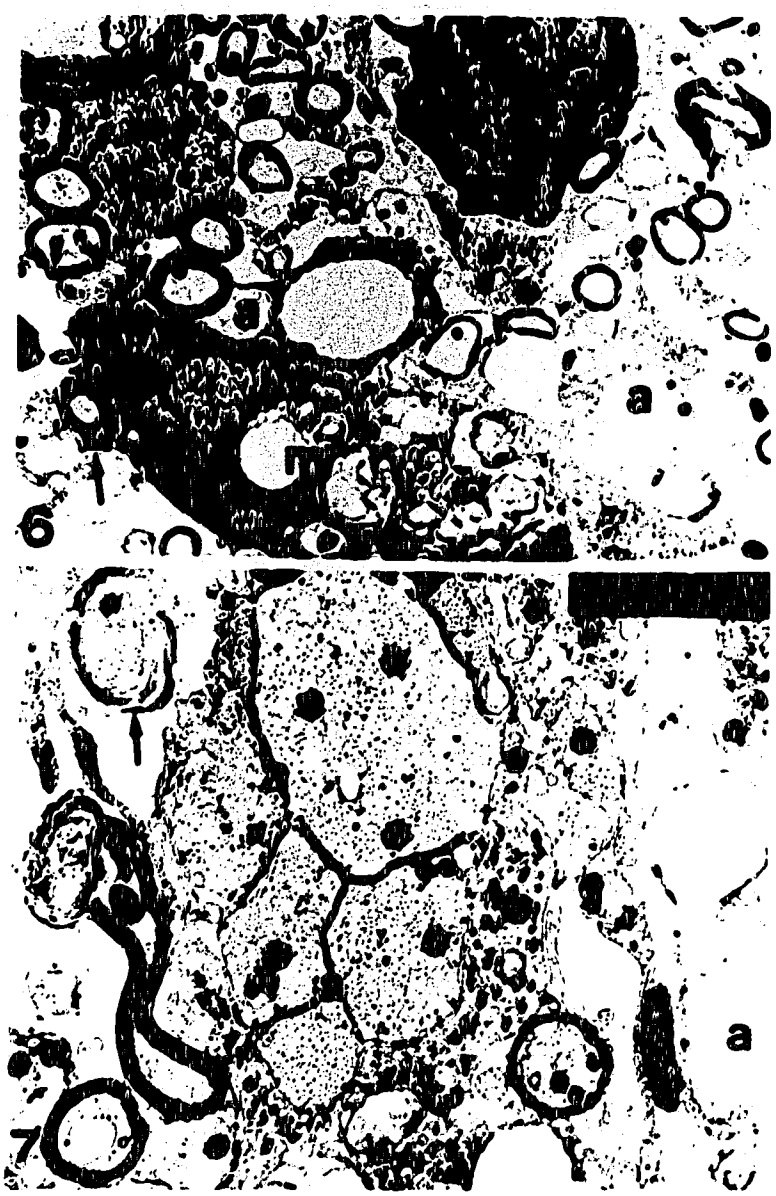

a
a

Figure 3.8 Subpial area in the lumbar spinal cord shows numerous demyelinated axons (a) and a macrophage (m). The subarachnoid space on the left shows a mononuclear cell at the bottom and a polymorphonuclear inflammatory cell at the top. The area shown by an asterisk is presented by higher magnification in Fig. 3.9 (X2800).

Figure 3.9 A higher magnification from Fig. 3.8 shows a bare axon (a) and free floating myelin deside this axon on the left. Additionally, the cytoplasm of the cell on the right contains many microtubules (arrowheads) but no intermediate filaments indicative of an oligodendroglial-like cells. On the left an astrocytic cell process contains a dense accumulation of glial filaments (arrows) (X11,000).

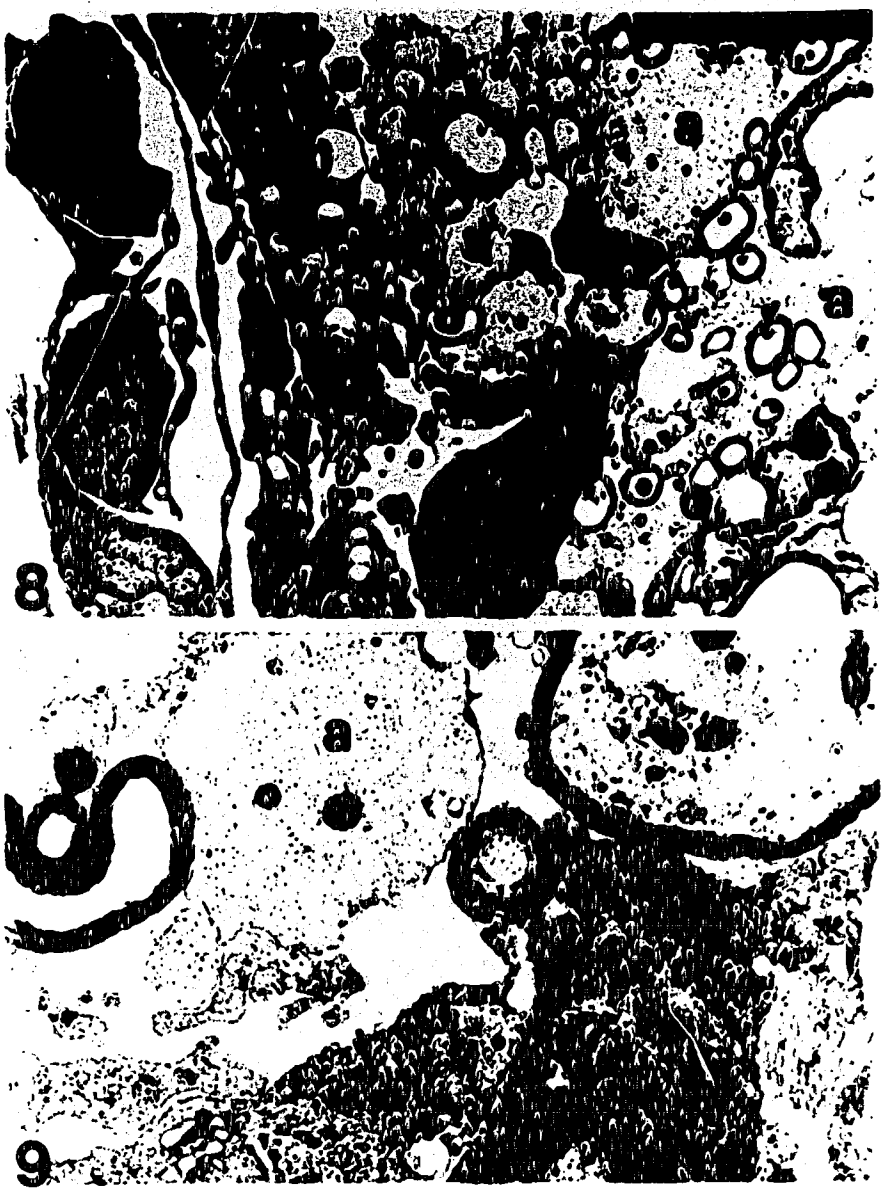

a
8
a
9

Table 4: Effect of Time of A7-SFV Infection on the Induction and Development of EAE

Exp.	Day p.i. MSCH[b]	mice with EAE/ Total mice	EAE onset (%)	Day EAE onset (p.i.MSCH)	Neurologic signs
I	3	0/10	0	-	-
	5	2/10	20	15	++ to ++++
	7	7/10	70[c]	13 - 14	+ to ++++
	9	2/10	20	15 - 16	++ to ++++
	mock infected	2/10	20	25	++ to ++++
	virus only[a]	1/10	10	16	++
II	4	2/10	20	11	++++
	5	2/10	20	11	++++
	6	6/10	60	12 - 14	+ to ++++
	7	8/10	80[d]	12 - 14	+ to ++++
	8	6/10	60	14 - 16	+ to ++++
	mock infected	3/10	30	24 - 26	+ to +++
	virus only	1/10	10	16	+++

a: Total of 10^6 PFU/mouse of A7-SFV was inoculated.
b: Mouse spinal cord homogenate.
c: Significantly different from other groups ($p < 0.05$).
d: Siginificantly different from day 4 and 5 ($p < 0.05$).

group), the rate of disease onset was about 20 - 30%. One out of ten mice treated with 350 R and virus infection developed paresis. The potentiation effect of virus infection at day 7 post neuroantigen inoculation was significantly higher than at day 4 or 5 ($p < 0.001$). No significant difference was observed in the case of time of disease onset among different groups.

Effect of Infectious Dose of A7-SFV on EAE Development

Different amounts of A7-SFV were inoculated i. p. at day 7 post neuroantigen inoculation. Injection of 10^6 PFU/mouse had an optimal potentiation effect, about 70 - 80% mice treated with neuroantigen and virus infection developed neurological signs of EAE on average at day 14 (Table 5). An amount of 10^2 or 10^4 PFU per mouse did not increase the incidence of EAE onset but did shorten the time of disease onset (average of 14 days post neuroantigen inoculation). An inoculum of 10^8 PFU per mouse did not enhance the incidence but did again shorten the time of disease onset. The potentiation effect of 10^6 PFU inoculum was significantly higher than that of other groups ($p < 0.01$).

Effect of Inactivated A7-SFV on the Development of EAE

To investigate whether virus infection is needed for the observed potentiation effect on EAE development, the SFV virus suspension was UV inactivated and 10^6 PFU inactivated virus per mouse was inoculated i. p.. No potentiation

Table 5: Effect of Infectious Dose of A7-SFV on the Induction and Development of EAE in BALB/c Mice

Virus Inoculum (PFU/mouse)[c]	EAE mice/ Total mice	EAE Onset (%)	Time of Disease Onset	Neurological Signs
0	7/20	35	24 - 26	++ to +++
10^2	4/15	27	13	++ to ++++
10^4	3/15	20	14	++
10^6	11/15	73[d]	12 - 13	+ to ++++
10^8	3/15	20	15	++
10^{6a}	2/20	10	16[b]	+

a: No mouse spinal cord homogenate (MSCH) was inoculated.
b: Time p.i. mock inoculation of MSCH.
c: Virus was inoculated day 7 after MSCH inoculation.
d: Significantly different from other groups ($p < 0.01$)

effect was found in the group of mice treated with neuroantigen and inactivated virus (Table 6) and about 30% of mice developed neurologic signs of EAE at day 21 - 24 after neuroantigen inoculation, which was the same as from the group of mice treated by irradiation and neuroantigen only. It was concluded, therefore that virus infection was needed for the potentiation effect.

Effect of A7-SFV Infection Before Neuroantigen Inoculation on EAE Induction in BALB/c Mice

The potentiation effect of SFV infection was time and dose dependent (Table 4, 5). It has also been reported by others that the temporal relationship of virus infection and induction of EAE is an important factor for the outcome of the experiments (101, 102). To further test this, virus infection was given before the induction of EAE. Twenty mice were infected with A7-SFV 21 days before neuroantigen inoculation, and at the same time, another twenty mice were treated with the same regimen except that 350 R irradiation was introduced two days before neuroantigen inoculation (Table 7). In both cases, none of the mice developed EAE. Predisposing of BALB/c mice to SFV infection actually prevented the disease onset. In the control groups, about 30% of mice in the group treated with 350 R and neuroantigen, and 90% of the mice in the group treated with 350 R, neuroantigen and virus developed neurologic signs of EAE.

Table 6: A7-SFV Infection Is Required for the Potentiation of the EAE Development

gp	Regime	EAE mice/ Total mice	EAE onset (%)	Day EAE onset (p.i.MSCH)	Neurologic sign
1	IR+MSCH+PV+UV-A7[a]	3/10	30	21 - 24	+++ to ++++
2	IR+MSCH+PV+A7	9/10	90	12 - 14	+++ to ++++
3	IR+MSCH+PV	4/10	40	21 - 26	+++ to ++++
4	IR+PV+A7	1/10	10	16	++

a: IR: 350 irradiation. MSCH: mouse spinal cord homogenate. UV-A7: U.V. inactivated A7-SFV. PV:pertussis vaccine.

Table 7: Effect of A7-SFV Infection 21 Days before EAE Induction in Non-irradiated BALB/c Mice

Regime	mice with EAE/ Total mice	EAE onset (%)	Day of EAE onset[e]	Neurologic signs
A7[a]+MSCH+PV	0/20	-	-	-
A7 +IR+MSCH+PV[b]	0/20	-	-	-
A7	0/20	-	-	-
IR+MSCH+PV+A7	9/10	90[d]	12 - 14	+++ to ++++
IR+MSCH+PV	3/10	30	24 - 25	+++ to ++++
IR+PV+A7	1/10	10	17 - 18[c]	+ to ++

a: Total of 10^6PFU/mouse of A7-SFV was inoculated i.p. 21 days before MSCH inoculation.
b: IR: 350 irradiation. MSCH: mouse spinal cord homogenate. PV: pertussis vaccine.
c: Day after mock-inoculation of MSCH (inoculation of PBS).
d: Significantly different from other groups ($p < 0.001$).
e: P.i. of MSCH.

In a separate protocol, both SJL/J and BALB/c mice were infected 12 days before neuroantigen inoculation (10 days before 350 R irradiation in BALB/c mice) (Table 8). About 60% of SJL/J mice inoculated with neuroantigen developed EAE. However, only 10% developed EAE in the group of mice infected 12 days earlier, and virus by itself did not cause clinical disease in SJL/J mice. The same prevention effect can be observed in BALB/c mouse strain as well. Therefore, the down regulation effect caused by antecedent virus infection was true for both SJL/J and BALB/c mouse strains. This phenomenon was not further studied in this work.

Observations of Spleen Weight Changes in Mice Treated with Different Regimens

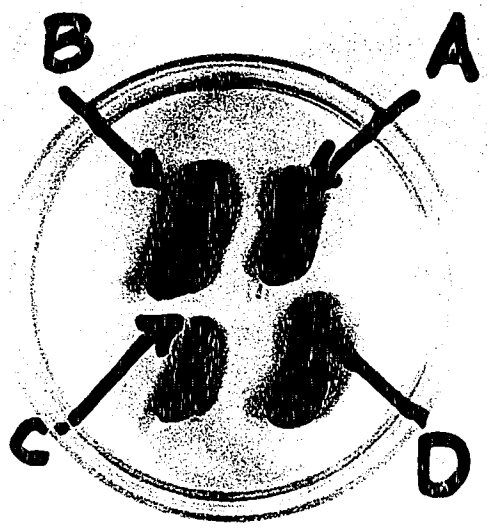
Whole body irradiation with 350 R reduced spleen size but the sign returned to normal at day 9 - 10 days if the animals were not infected. The spleen size of the animals with virus infection alone remained reduced for a longer time but recovered to normal about day 14 after irradiation. Spleen atrophy remained through the observation period in the BALB/c mice injected with MSCH and infected with SFV 7 days later and with or without clinical signs of EAE (Fig.4). On the other hand, animals with MSCH inoculation alone had swollen spleens until day 26 after which the spleen size of the animals developing EAE started to decrease. Reduced spleen weight in mice

Table 8: Effect of A7-SFV Infection 12 Days before Injection of MSCH on EAE in BALB/c and SJL/J Mice[a]

Mouse Strain	EAE Induction Regime[b]	EAE Mice	Clinical Assesment
BALB/c	V+IR+MSCH	0/30	-
	IR+MSCH	7/30	++ to ++++
	V+IR	0/30	-
SJL/J	V+MSCH	1/10	+++
	MSCH	6/10	+++
	V	0/10	-

a: Preinfection with SFV 10 days before irradiation and 12 days before injection of MSCH.
b: V. A7-SFV, 10^6 PFU was injected to each mouse i. p. MSCH. mouse spinal cord homogenate. IR. irradiated with 350 R.

Figure 4 Spleens taken from BALB/c mice treated with different regimens at day 20 after mock- or MSCH inoculation. A. Normal mouse; B. Mouse treated with 350 R and MSCH; C. Treated with 350 R, MSCH and virus; D. Treated with 350 R and virus.


B
A
C
D

treated with both MSCH and virus may not reflect a decreased number of spleen cells. At day 12 after MSCH inoculation, namely, day 5 after virus infection, there was no substantial difference in case of numbers of spleen cells harvested between only MSCH inoculated mice and mice treated with the combined regimen. These findings suggest that general immunological changes are associated with the enhancement of EAE in the animals.

Reactivity of Lymphocytes from Mice Treated with Different Regimens to ConA Stimulation

ConA stimulation of spleen cells was used as a test for the general T cell response *in vitro*. The blastogenic response of spleen cells from animals treated with neuroantigen only was suppressed at days 13 and 14, the time when the early onset of demyelination was seen in the neuroantigen plus SFV group, whereas virus infection alone caused a slight depression in this test (Table 9). The group with combined treatment had a variable but on the average a normal reactivity in the ConA stimulation.

Reactivity of Lymphocytes from Mice Treated with Different Regimens to Antigen Stimulation

Lymph node cells were prepared from five mice in each group treated with different regimens at day 9, 14 and 15 after neuroantigen inoculation, and stimulated with MBP, PLP or inactivated SFV antigen. At day 9 after neuroantigen

Table 9: Reactivity of Spleen Cells to ConA Stimulation at Days 13 and 14 After Injection of the MSCH[a]

		Incorporated Radioactivity (Mean ± S. D.)			
		Control	MSCH+SFV	MSCH	SFV
Exp. 1	Day 13	45802 ± 3892	29277 ± 2009	11831 ± 2338	47228 ± 2083
	Day 14	53357 ± 2536	38489 ± 5330	15489 ± 1330	34215 ± 661
Exp. 2	Day 13	30927 ± 1667	36107 ± 2125	18432 ± 2357	28030 ± 2856
	Day 14	32326 ± 1902	31044 ± 1613	21340 ± 1379	25108 ± 249

a. Three randomly selected animals were tested in each group.

inoculation, lymphocytes from all groups proliferated to MBP. Only cells from mice treated with neuroantigen or the combined regimen responded to PLP. Cells from mice treated with both neuroantigen and virus or virus alone responded to viral antigen. The cells from mice infected with virus alone had the best response to viral antigen (Table 10). At day 15 after neuroantigen inoculation, the responses to MBP, PLP and SFV antigen remained in the group of mice with the combined treatment. Nevertheless, the response to MBP or PLP was decreased in the group of mice treated with either neuroantigen or A7-SFV alone at day 15 after neuroantigen inoculation.

Phenotype Markers of Immune Cells in Spleen and Lymph Node after Neuroantigen Inoculation and/or A7-SFV Infection

Flow cytometry was used to analyse the composition of different cell populations after neuroantigen inoculation and/or virus infection (Table 11). In order to analyse the cells prior to, at the beginning and during the peak of the EAE, the analysis was done at days 9, 11 and 15 after neuroantigen inoculation. When cell distribution was compared in neuroantigen inoculated and normal mice, the following differences were observed: L3T4+ T cells were decreased at all observed times; Lyt. 2+ T cell numbers were not different from normal mice at days 9, 11 after neuroantigen injection, but at day 15 they were lower than that in uninfected mice; Ia+ cells remained higher at all

TABLE 10. Antigen Driven Proliferation of Lymphocytes from Balb/c Mice Treated with Differenct Regimens In Vivo

Treatment in vivo[a]		Time p.i. MSCH	cpm ± S.D.[b]				S.I.[c]		
MSCH	A7-SFV		MBP	PLP	SFV Antigen	Medium	MBP	PLP	SFV Antigen
Ex. 1									
+	-	9	2244 ± 64	1628 ± 40	1976 ± 150	819 ± 123	2.7	2.0	2.4
+	+	9	1716 ± 103	1253 ± 37	1771 ± 140	540 ± 52	3.2	2.3	3.3
-	+	9	1127 ± 174	378 ± 18	1816 ± 145	218 ± 22	5.2	1.7	8.3
+	-	15	75 ± 14	134 ± 21	103 ± 17	1 22 ± 34	0.6	1.1	0.8
+	+	15	3699 ± 272	2714 ± 361	4701 ± 665	1718 ± 324	2.2	1.6	2.7
-	+	15	3049 ± 329	1026 ± 112	4709 ± 631	1543 ± 254	1.9	0.7	3.1
+	-	9	2549 ± 38	2010 ± 78	1804 ± 157	942 ± 87	2.7	2.1	1.9
+	+	9	2010 ± 207	1542 ± 181	2072 ± 341	706 ± 67	2.8	2.2	2.9
-	+	9	1027 ± 170	490 ± 71	1970 ± 201	403 ± 40	2.5	1.2	4.9
+	-	14	170 ± 24	120 ± 17	154 ± 71	270 ± 25	0.6	0.4	0.6
+	+	14	4042 ± 408	3248 ± 541	5041 ± 687	1540 ± 148	2.6	2.1	3.3
-	+	14	2740 ± 320	9 87 ± 107	4981 ± 78	1468 ± 149	1.9	0.7	3.4

a: All animals received 350 R and pertussis vaccine as well. b: See Materials and Methods for details. Cells are pooled from five animals in each day in each group. The responses to antigens of cells from normal mice were usually between 200 to 400 cpm/miin.
c: S.I.: stimulation Index = cpm of antigen per cpm of medium

Table 11: Cellular Composition of Mouse Spleen and Lymph Node cells after Neuroantigen inoculation and/or A7-SFV Infection *in vivo*[a]

Day p.i.	Regimen[b]	Ex.	Lymph node						Spleen					
			L3T4+	Lyt.2+	Ia+	Mac-1+	mIg+	ratIg	L3T4	Lyt.2+	Ia+	Mac-1+	mIg+	rat Ig
9	Normal mice (C)	I	36.3	25.7	9.8	8.9	10.2	6.5	15.1	17.5	20.7	6.9	33.7	10.6
	Neuro-antigen (N)	I	13.9	19.5	22.6	21.0	33.2	7.4	27.4	23.3	41.1	27.5	46.1	10.9
		II	15.1	23.4	24.6	25.0	36.0	9.2	28.1	25.3	44.0	30.2	47.0	10.2
	N+A7	I	48.1	20.6	23.5	14.1	16.9	12.2	26.8	22.4	40.5	29.3	49.8	16.5
		II	72.4	15.4	15.3	16.8	19.0	11.3	29.2	21.4	40.2	27.4	48.0	13.4
	A7	I	19.4	15.9	10.6	20.6	9.0	7.1	19.5	20.2	18.6	45.9		4.9
		II	20.4	15.6	12.4	21.2	10.0	8.4	18.6	19.9	20.6	44.6	40.4	5.6
11	C	I	36.6	25.4	8.2	4.9	8.7	4.4	14.7	15.6	22.2	12.3	38.6	9.7
	N	I	18.8	26.7	26.1	17.0	35.8	12.4	12.0	13.0	21.0	17.1	26.8	10.6
		II	20.1	27.2	26.4	18.0	39.8	10.7	14.0	15.6	24.0	18.6	30.7	11.2
	N+A7	I	35.5	14.0	12.4	9.5	16.9	7.6	16.0	14.3	24.9	19.3	30.6	13.6
		II	36.0	16.0	14.2	10.4	15.8	8.2	15.0	16.4	25.6	20.1	32.6	11.4
	A7	I	59.1	19.1	11.0	11.5	14.5	10.9	15.1	14.2	29.3	14.4	37.6	10.2
		II	65.0	26.0	15.3	14.3	17.9	13.8	16.4	15.2	27.4	15.3	39.2	11.0
15	C	I	42.0	30.7	12.2	8.4	10.1	9.8	14.5	16.0	23.7	13.4	38.3	10.6
	N	I	9.1	13.4	32.8	11.1	37.1	11.6	5.1	4.3	17.8	9.3	23.6	6.8
		II	10.6	15.3	38.2	13.4	39.2	10.4	6.1	7.9	19.8	11.4	25.6	7.8
	N+A7	I	59.8	24.0	17.8	21.6	17.6	17.2	13.2	10.1	20.6	13.0	32.4	10.2
		II	45.2	18.2	14.2	16.7	16.7	10.0	15.4	14.2	24.7	14.0	30.2	9.8
	A7	I	79.9	14.2	16.9	9.6	23.3	9.2	13.3	12.3	22.0	10.9	33.3	8.7
		II	51.5	22.0	17.7	12.0	17.0	11.5	14.6	14.7	24.2	12.0	35.4	9.7

a. Cells were pooled from five mice in each day in each regimen. Total of 10^6 PFU of A7-SFV per mouse was inoculated at day 7 after neuroantigen inoculation, therefore, day 9 after neuroantigen injection indicates day 2 after virus infection. Percentages of cells positive for L3T4, Lyt.2, Ia, Mac-1 or mIg determined by FACS analysis are shown. b: Neuroantigen: mice irradiated with 350 R and inoculated with MSCH. N+A7: Mice irradiated with 350 R, inoculated with MSCH and infected with A7-SFV. A7: mice irradiated with 350 R and infected with A7-SFV.

observed time and so did B cells; A higher level of Mac-1+ cells was also found at days 9 and 11 after neuroantigen inoculation, but the level returned to normal at day 15.

The cellular composition in lymph nodes from mice inoculated with both neuroantigen and A7-SFV was different: A transient increase of L3T4 T cells was shown at day 9 after neuroantigen inoculation, namely, 2 days after virus infection. Thereafter, the relative number of L3T4 cells returned to normal; Lyt. 2+ T cells decreased at day 11 and day 15; Ia+ cells increased at day 9 and returned to normal afterwards, as did Mac-1+ cells; the B cell number was the same as in normal mice. In the mice treated with 350 R and virus, low levels of L3T4 T cells were found at day 9, and day 11 after mock inoculation of neuroantigen, but at day 15, L3T4+ T cells increased to a higher level than that of normal mice; The number of Lyt. 2+ T cells was the same as in normal mice at day 9, but increased at day 11; The number of Ia+ cells and B cells was about the same as normal mice; the Mac-1+ cell number was increased at day 7, but returned to normal afterwards.

Cellular composition in spleens was different from that in lymph nodes. In spleens of neuroantigen inoculated mice, L3T4+ T cells increased at day 9, returned to normal at day 11, then decreased at day 15; Lyt. 2+ T cells remained normal at day 9, 11, but decreased at day 15; Ia+ cell number increased at day 9, returned to normal at day 11, and 15, as did Mac-1+ cell number; No difference in B cells

was found between this group and normal mice. In mice treated with both neuroantigen and virus, both L3T4+ and Lyt. 2+ T cell number were the same as in normal mice; Ia+ cell number increased at day 9, and returned to normal afterwards, as did Mac-1+ cell number; the B cell number was at same level as in normal mice. The same pattern of cellular composition was observed in the group of mice infected with A7 only.

Taken together, these experiments show that a complicated redistribution of immune cells was observed among the three groups of mice treated with neuroantigen, and/or infected with virus as well. However, the results are difficult to interpret and their relationship to the enhancement of EAE could not be determined.

Effect of In vivo Administration of Antibodies to Helper T Cell Antigen L3T4, Suppressor T Cell Antigen Lyt.2 and Monocyte/Macrophage Antigen F4/80 on EAE Induction

$L3T4^+$ T cells and macrophages are known to be important for EAE induction in numerous EAE models (2). Since an additional element, virus infection, was introduced into this model, it was necessary to investigate if these two cell populations are also involved in disease induction in this model. *In vivo* administration of monoclonal antibody (MAb) against different phenotypes of immune cells was chosen because it has been shown to be an efficient way to deplete certain subpopulations of immune cells (25, 204).

As shown in Table 12, either *in vivo* administration of anti-L3T4 MAb or anti-L3T4 and anti-Lyt.2 Mab prevented BALB/c mice from developing neurological signs of EAE. Similarly, administration of F4/80 MAb, which reacts with resident and responsive macrophages (187), reduced the incidence of clinical EAE. No prevention effect was observed with administration of anti-Lyt. 2 MAb. An unexpected result , among all groups receiving MAbs, was that about 20 - 40% of mice died about 4 - 5 days after receiving MAbs. Since it is known that both T and macrophages play critical roles in anti-SFV immunity (136, 162, 163, 164), *in vivo* administration of anti-L3T4, anti-Lyt. 2, or F4/80 may decrease the immunity against SFV infection and therefore, avirulent virus could become more pathogenic. To investigate this possibility, BALB/c mice were irradiated with 350 R , and treated only with MAbs with or without virus infection (Table 12). The same incidence of death was found among all groups treated with MAb and virus, but not with MAb only. It was concluded, therefore, that *in vivo* administration of anti-L3T4, or F4/80 MAbs can prevent EAE onset, but anti-Lyt. 2 MAb did not have such an effect. Nevertheless, *in vivo* administration of anti-L3T4, or anti-Lyt. 2 or F4/80 MAb can increase the virulence of A7-SFV.

Table 12: Effect of *In Vivo* Administration of Anti-L3T4 and/or Anti-Lyt.2 MAb on EAE Potentiated by SFV Infection in BALB/c Mice

gp.	MSCH	A7-SFV	Injection of MAb[a]				Death/ Total mice	EAE mice/ Total mice
			Anti-L3T4	Anti-Lyt.2	F4/80	Control Ab		
1	+	+	-	-	-	-	0/10	8/10
2	+	+	+	-	-	-	3/10	1/7[c]
3	+	+	-	+	-	-	2/10	5/8[d]
4	+	+	+	+	-	-	4/10	0/6[e]
5	+	+	-	-	+	-	2/10	2/8[f]
6	+	+	-	-	-	+	0/10	8/10
7	-	+	+	-	-	-	3/10	0/7
8	-	+	-	+	-	-	3/10	0/7
9	-	+	+	+	-	-	3/10	0/7
10	-	+	-	-	+	-	2/10	0/8
11	-	+	-	-	-	-	0/10	1/10
12	-	-	+	-	-	-	0/10	0/10
13	-	-	-	+	-	-	0/10	0/10
14	-	-	+	+	-	-	0/10	0/10
15	-	-	-	-	+	-	0/10	0/10

a.0.3 ml MAb was injected into each mouse i.v. five days after MSCH inoculation. Gk. 1.5 is a MAb which recognize L3T4 molecule on T helper cells; 53.6.72 is a MAb which recognize Lyt.2 molecule on T suppressor/cytotoxic cells; F4/80 is MAb which recognize the antigen on resident and responsive macrophages. Normal rat IgG was used as control Ab. b.Death occurred at day 2-4 after virus injection. c: significant different from group 1 $p < 0.05$. d: not significantly different from group 1. e: significantly different from group 1, $p < 0.025$. e: significantly different from group 1, $p < 0.05$.

Chapter Three

Replication of Semliki Forest Virus in BALB/c Mice *in vivo* and *in vitro*

Infectious A7-SFV in Blood and Brain of Infected Mice

As A7-SFV infection is an essential part of the model, it is necessary to reveal the replication kinetics *in vivo*. Blood samples from 5 BALB/c mice or 3 SJL/J mice were pooled in each experiment each day. Sera were titrated on the MBA-13 cell line by a microtitration assay, and the infectious virus titer was expressed as $TCID_{50}$/ml. A7-SFV infection of either BALB/c or SJL/J mice caused a transient viramia (Table 13). Peak titer of virus was observed at 24 h after infection, and it decreased afterwards. At day 4 p. i., no infctious virus was detected. No significant difference in virus titers was found between the two mouse strains.

BALB/c mouse brains were taken at different days after A7-SFV infection, homogenized in PBS, and centrifuged by high speed centrifugation. The supernatants were titrated in the MBA-13 cell line by a microtitration assay. Low levels of infectious virus was observed (Table 14). Virus replication peaked at day 4 or 5 after infection. High

Table 13: Infectious A7-SFV in Blood of BALB/c and SJL/J Mice[a]

Day p.i.[b]	Infectivity (log $TCID_{50}$/ml)	
	BALB/c	SJL/J
1	7.0	7.5
2	6.5	6.5
3	4.0	4.5
4	0.0	0.0
5	0.0	0.0
6	0.0	0.0
7	0.0	0.0

a: Blood samples (0.5 ml blood per mouse) were pooled from 5 BALB/c mice or 3 SJL/J mice in each day. b: 10^6PFU/mouse of A7-SFV was inoculated i.p.

Table 14: Infectious A7-SFV in BALB/c Mouse Brains[a]

Day p.i.[b]	Infectivity (log $TCID_{50}$/g)	
	Ex. 1	Ex. 2
1	3.0	3.5
2	2.0	1.5
3	2.0	2.0
4	3.5	3.0
5	3.5	3.3
6	2.0	2.0
7	1.5	1.5
8	0.0	0.0
9	0.0	0.0
10	0.0	0.0
14	0.0	0.0

a: Brain homogenates were pooled from five mice in each day.
b: Total of 10^6PFU/mouse of A7-SFV was inoculated.

virus titers were also found at day 1 p.i. but those could possibly be explained by the input virus. Virus infectivity decreased gradually after day 5 p.i., and became undetectable at day 8 p. i. Infectivity titrations were carried out until day 14 p. i., but no infectious virus was detected any more.

Effect of 350 R Irradiation on A7-SFV Replication in Mice

Low dose whole body irradiation of BALB/c mice two days before infection not only increased but also prolonged the presence of A7-SFV in blood (Table 15). Viremia lasted about 3 days longer than in nonirradiated mice (Table 13, 15). Infected mice cleared the virus at about day 8 p. i. When mice were infected at day 7 p. i., viremia was similar as found in mice without irradiation.

BALB/c mice were infected with A7 virus at day 2 after low dose whole body irradiation. Irradiation had no effect on virus replication in the brain the first three days after virus infection (Table 16). About one log higher virus titer was observed day 3 p. i. (Table 14, 16). Virus titers reached peak levels at day 4, 5, and decreased afterwards, but presence of virus was prolonged to day 10 p. i.. In the mice infected with A7-SFV at day 7 after 350 R irradiation, the virus titer kinetics were the similar as in nonirradiated mice.

Replication of SFV in Splenocytes in vitro

SFV infection of different immune cell populations were carried *in vitro*. As it has been suggested that virulence of different SFV strains may be related to infectivity (95), W-SFV was also included in the *in vitro* investigation. Splenocytes and fractions enriched for adherent (ad+) or nonadherent (ad-) cells were infected with W-SFV and A7-SFV at different m.o.i. and the expression of viral antigen was determined by immunofluorescence. A dose dependent increase in the number of antigen positive cells was observed with both SFV strains. The W-SFV infected a higher percentage of the spleen cells than the A7-SFV at all multiplicities of infection (Fig. 5). At a m.o.i. of 64, only 6.5% of splenocytes infected with the A7-SFV were positive for virus antigen at 48 h after infection as compared to 18.5% of the cells infected with the W-SFV strain.

A higher proportion of the ad+ cells were positive for SFV antigen than the ad- cell population (Table 17). Double immunofluorescence revealed that the adherent cells infected with SFV were Mac-1+ cells. All cells were incubated for 6 days in a 5% CO_2 atmosphere at 37° C, and supernatants harvested daily and titrated. Neither splenocytes nor separately infected ad+ or ad- cells released any infectious virus. Therefore, it was concluded that SFV infection of splenocytes *in vitro* was an abortive type infection, and splenic macrophages (Mac-1+ cell population) the primary target.

Table 15: Infectious A7-SFV in Blood of BALB/c Mice Treated with 350 R

Day p.i. A7[b]	Infectivity (log $TCID_{50}$/ml)	
	Ex.1	Ex.2
1	8.5	8.0
2	7.0	7.5
3	6.0	6.5
4	5.5	5.0
5	4.0	4.0
6	3.0	3.0
7	1.5	1.0
8	0.0	0.0

a: Blood samples (0.5 ml per mouse) were pooled from 5 BALB/c mice in each day.

b: 10^6 PFU/mouse of A7-SFV was inoculated i.p..

Table 16: Infectious A7-SFV in Brains of BALB/c Mice Irradiated with 350 R

Day p.i.[b]	Infectivity (log $TCID_{50}$/g)[a]	
	Ex.1	Ex.2
1	3.5	3.5
2	2.0	2.0
3	2.0	2.5
4	4.5	4.5
5	5.0	4.5
6	3.5	3.0
7	2.0	2.0
8	2.0	1.5
9	1.5	1.5
10	0.0	1.0
14	0.0	0.0

a: Brain homogenates were pooled from five mice in each day.
b: 10^6 PFU/mouse of A7-SFV was inoculated.

Figure 5. Dependence of spleen mononuclear cell infection by SFV strains on the multiplicity of infection. Cells were infected at different multiplicities and the percentage of SFV antigen positive cells was determined by indirect immunofluorescence at 48 h after infection. A7 = infected with A7-SFV. W = infected with wild type SFV.

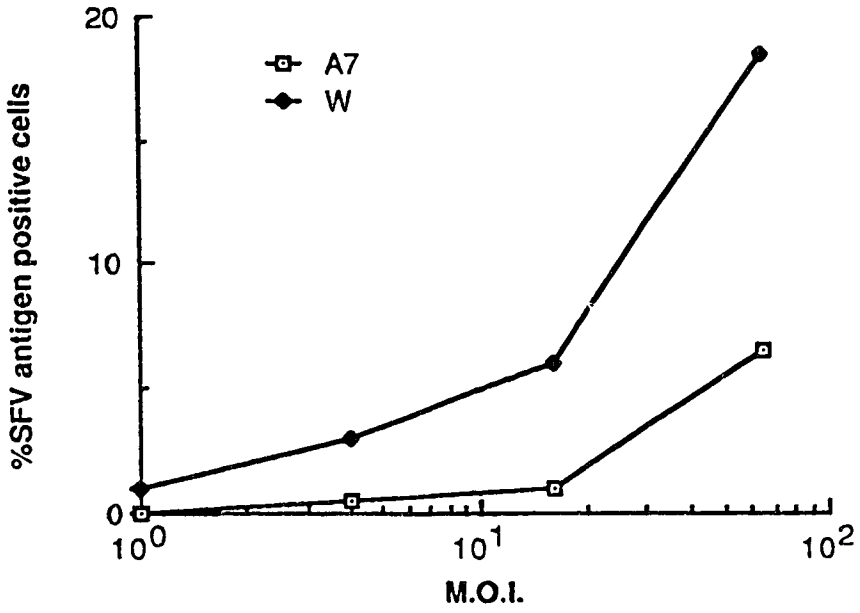

20
10
0
A7
W
%SFV antigen positive cells
10^0
10^1
10^2
M.O.I.

Table 17: Detection of SFV antigen in ad^+, ad^-, Mac-1^+ and Mac-1^- splenocytes 48 h after infection. The percentage of cells positive for SFV antigen in these populations are shown.

Virus strain	m. o. i.	% of SFV positive cells in spleen cell population			
		ad^+	ad^-	Mac-1^+	Mac-1^-
W-SFV	10	14.3	3.0	20.0	1.7
	100	71.4	2.7	N.D.[a]	N.D.
A7-SFV	10	8.0	2.0	12.5	0.5
	100	32.3	2.5	N.D.	N.D.

a. Not done.

Replication of SFV in Murine Peritoneal Macrophages In vitro

Thioglycollate elicited peritoneal macrophages (PM) were infected by A7-SFV. The macrophages cleared virus completely by 6 days after infection (Table 18). Only 5% of macrophages was infected as determined by immunofluorescence detection of viral antigen synthesis which was correlated with production of infectious virus particles peaking at 24 h p. i.. At the peak level, only one infectious virus particle was produced per 10 macrophages. Through the entire observation period, no cytopathic effect (CPE) was observed. As the residual cell membrane associated virus had been neutralized by antiserum, the calculated virus titer should represent newly produced virus.

A7-SFV Infection of Bone Marrow Derived Macrophages (BMDM) at Different Development Stages

Bone marrow derived macrophages were fractionated by fetal bovine serum gradient sedimentation. Forty fractions were collected and pooled into four larger fractions according to the

size of macrophages (169, 170). Fraction A represented immature, smallest size of macrophages in BMDM. Thereafter, the size increases gradually. Fraction D therefore represents the mature macrophages. Different fractions of

Table 18: A self Limited Replication of A7-SFV on Murine Thioglycollate Elicited Peritoneal Macrophages (PMs)[a]

Day p.i.	Released infectious virus ($logTCID_{50}$/ml) from $2X10^6$cells	SFV antigen positive cells(%)
0	0.6±0.8	0.0
1	4.0±0.5	4.8±0.9
2	3.5±0.1	2.4±0.4
3	3.2±0.3	2.0±0.1
4	0.8±0.1	0.3±0.5
5	0.8±0.1	0.2±0.2
6	0.0	0.0
7	0.00	0.00

a: Mean and S. D. from three separate experiments

BMDM were infected with A7-SFV at m.o.i. of 10, and viral antigen synthesis and production of infectious virus were determined at 24 h post infection (Table 19). In non-fractionated BMDM, about 15% of cells were SFV antigen positive as determined by immunofluorescence with polyclonal anti-SFV antiserum.

A small number of infectious virus was produced 24 h after virus infection. Since these infected BMDM had been treated with SFV polyclonal antiserum at 4°C for 1 h, infectious virus could not be membrane associated residual input virus. Fraction A was the most resistant to SFV infection, only a small percentage of cells had virus antigen and no production of infectious virus was detected. An increased expression of viral antigen was found in fractions B, C and D. Viral antigen was detected in 18% of BMDM fraction D. No significant difference in production of infectious virus between fraction B, C and D was found. These data indicate that SFV infection is associated with the differentiation stage of BMDM.

Effect of Activation of BMDM on A7-SFV Replication

To investigate whether virus infection is related to the activation stage of macrophages, non-fractionated total BMDM and fraction C from experiments described above were treated with 10% rat ConA supernatant 24 h before virus infection. Rat ConA supernatant has been shown in preliminary experimants to be a good stimulus of

Table 19: Association of SFV infection with the differentiation stage of bone marrow derived macrophages (BMDM)[a,b]

cells	production of infectious virus (log $TCID_{50}$/ml from $2x10^6$ cells) 24 h p. i.	SFV antigen positive cells (%)
total cell population	2.4 ± 0.3	15.1 ± 0.2
fraction A	0.0	2.3 ± 0.1
fraction B	2.4 ± 0.2	5.4 ± 0.3
fraction C	2.6 ± 0.2	11.2 ± 0.3
fraction D	2.8 ± 0.4	18.3 ± 0.4

a: BMDM were prepared from BALB/c mice by flushing out bone marrow with medium. After 6 days incubation at 37°C, cells were fractionated by FBS gradient sedimentation and seeded to plates with the same surface area. The size of macrophages increased gradually from fraction A to fraction D, which correlated with the differentiation state of macrophages.
b: Mean ± S. D. from 3 separate experiments.

macrophages for Ia antigen expression. No difference was observed between activated and non-activated BMDM in total cell population or fraction C in viral antigen synthesis or production of infectious virus (Table 20). This suggests that SFV infection is not related to activation of bone marrow-derived macrophages. Further evidence was also obtained from PMs, as both Ia^+ or Ia^- macrophages were similarly infected after ConA activation (Fig. 6).

Detection of Viral RNA Synthesis in PM with SFV Probes

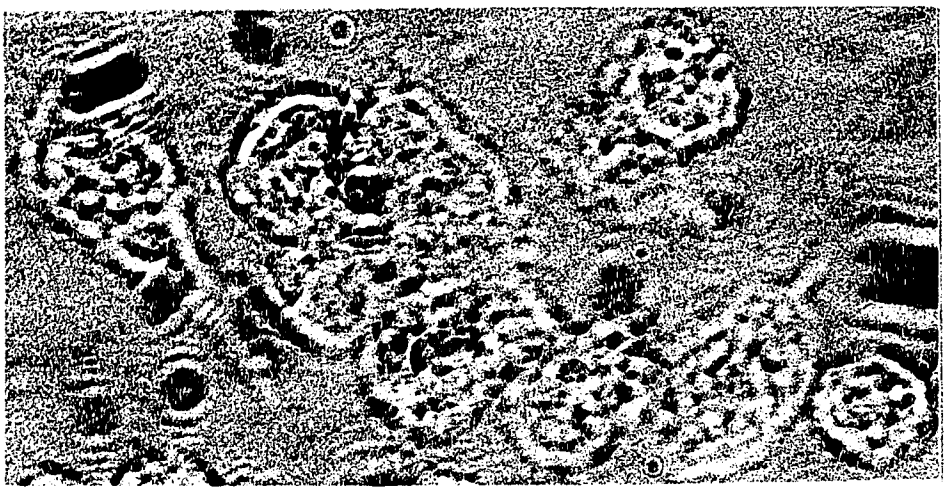
Even though both viral antigen synthesis and production of infectious virus was not detected with immunofluorescence and microtitration assay after virus infection, the possibility that viral RNA may be still synthesized but translation of transcripts was blocked in infected macrophages must be considered. Alternatively, virus RNA may persist inside host cells without active replication. To test these possibilities, total RNA was extracted from mock or infected macrophages at day 1, 4, 8 and 12 post virus infection. Viral RNA was detected with [^{32}P]-labelled probes which recognizes coding regions for viral membrane glycoprotein E1 or E2/E3. As shown in Fig. 7, the highest level of viral RNA was detected at day 1 after infection as demonstratd by a strong hybridizing band with the E3/E2 probe (Fig. 7, Lane A). Subsequently the intensity of the hybridization bands gradually decreased indicating the presence of a low amount of SFV RNA at day 8 and complete

Table 20: SFV infection is not related to the activation state of bone marrow derived macrophages (BMDM)[a,b]

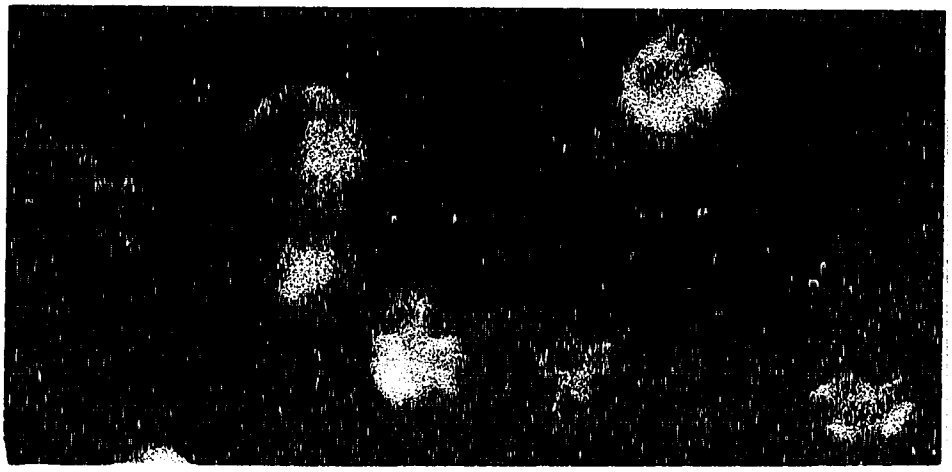
cells	production of infectious virus (log $TCID_{50}$/ml from $2x10^6$ cells) 24 h p. i.	SFV antigen positive cells (%)
activated total population	2.7 ± 0.1	15.0 ± 0.2
nonactivated total population	2.5 ± 0.2	12.1 ± 0.3
activated fraction C	2.6 ± 0.1	11.7 ± 0.6
nonactivated fraction C	2.7 ± 0.1	11.3 ± 0.5

a. BMDM were prepared from BALB/c mice by flushing out bone marrow with medium. After 6 days incubation at 37°C, cells were fractionated by fetal bovine serum (FBS) gradient sedimentation and seeded to plates with the same surface area. RPMI1640 complete medium containing 10% rat ConA supernatant was used to activate macrophages.
b. Mean ± S. D. from 3 separate experiments.

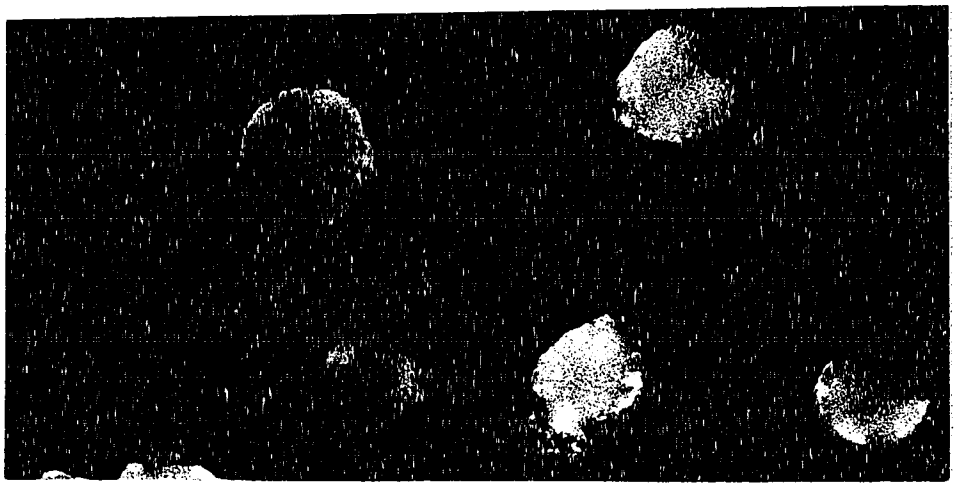
Figure 6. Peritoneal macrophages were infected by A7-SFV and stained for Ia and SFV antigen with a double immunofluorescence method. A. Stained with anti-Ia MAb. B. Stained with anti-SFV polyclonal antiserum. C. Phase contrast. X600.



C

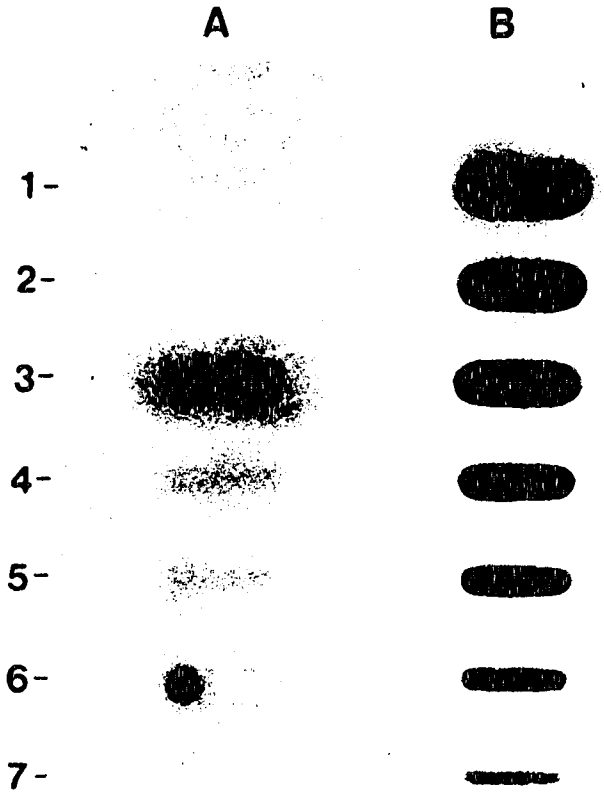


B



A

Figure 7. Slot blot analysis of RNA from thioglycollate elicited peritoneal macrophages. A 20 µg aliquot of total RNA from each of the samples was denatured in formaldehyde and formamide and spotted on GeneScreenPlus membrane and hybridized as described in Methods. Lane A: Total RNA extracted from mock infected peritoneal macrophages (Slot 1) and from macrophages infected with A7-SFV tested at days 1 (Slot 3), 4 (Slot 4), 8 (Slot 5) and 12 (Slot 6) after infection. Slots 2 and 7 are buffer blanks. Lane B: Positive control RNA samples. Twenty ug of total RNA isolated from SFV infected MBA-1 cells was denatured and a two-fold dilution series spotted to the membrane and hybridized (Slots 1-7). The data shown are from one of three repeated experiments with RNA samples from different batches using probes from the E3/E2 region of the SFV genome.

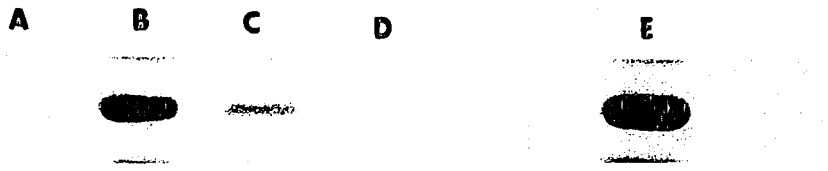

A
B
1-
2-
3-
4-
5-
6-
7-

absence of detectable RNA at day 12 after infection. The pattern of hybridization was similar when the blot was rehybridized with a probe from the E1 region. However the hybridization bands were less intense (data not shown) as compared to bands with the E3/E2 probe in infected macrophage samples.

Detection of Viral RNA Synthesis in Mouse Brains with A SFV Probe

Total cell RNA was extracted from mouse brains of mock- or virus-infected BALB/c mice at different times p. i. SFV RNA was detected with E1 or E3/E2 -probes as above. As shown in Fig. 8, most abundant viral RNA was detected at day 4 p.i. (Lane B), and a small amount of RNA was detectable at day 8 p.i.(Lane C). Afterwards, no viral RNA could be detected (Lane D). To exclude the possibility of viral persistence in the brain, mouse brain RNA was also extracted from A7-SFV infected mice at day 21 after infection. No viral RNA was detected. Therefore, this indicates that SFV does not persist in BALB/c mouse brains in this model.

Figure 8: Slot blot analysis of RNA from BALB/c mouse brains.A 40 µg aliquot of total RNA from each of the samples was denatured in formaldehyde and formamide and spotted on GeneScreenPlus membrane and hybridized as described. Lane A: Total RNA extracted from mock infected mouse brains. Lane B-D: Total RNA extracted from brains from infected mice at day 4(Lane B), 8(Lane C), and 12(Lane D) after infection. Lane E: Twenty µg of total RNA isolated from A7-SFV infected MBA-1 cells. The data shown was from one of three repeated experiments with RNA samples from different batches using probes E3/E2 region of the SFV genome.


A
B
C
D
E

Chapter Four

Transfer of EAE with *in vivo* Primed Spleen and Lymph Node Cells

Induction of EAE in Naive BALB/c Mice with Spleen or Lymph Node Cells from BALB/c Mice Treated with Different Regimens

The potentiation effect of virus infection on EAE development in the BALB/c mouse strain could be mediated virologically or immunologically. Virus infection may directly kill neurons and/or oligodendrocytes - cells in CNS responsible for remyelination. Virus may also kill endothelial cells, lining the vessels and participating in the blood brain barrier (BBB), thus altering the permeability of BBB. Neither case seems fit in this model, as A7-SFV does not kill these cells (159, 160). A question raised is whether this potentiation effect can be transferred to naive mice by primed cells.

Both spleen and lymph node cells were prepared from BALB/c mice treated with different regimens obtained at day 10 or 12 post neuroantigen inoculation. Cells were stimulated *in vitro* with ConA (usually 2.5 µg/ml for spleen cells, 5 µg/ml for lymph node cells) for 96 h at 37° C in a 5% CO_2 atmosphere. At this time, a trypan blue exclusion assay was done to determine the viability of the cultivated

cells. The viability was about 35 - 40% and was consistent in all experiments. Only lymph node or spleen cells from BALB/c mice treated with both neuroantigen and viral infection could induce the disease in naive animals, with about 20% of mice developing typical neurologic signs (hind limb paralysis, 2 - 3+) at day 12 after transfer (Table 21). Neither cells from mice inoculated with neuroantigen only nor cells from mice infected with virus only could induce a disease in naive mice. Both lymph node and spleen cells taken at day 10, or 12 post neuroantigen inoculation were able to transfer the disease. Of mice with neurologic signs , only two mice became moribound. An infectious center assay was used to detect infectious virus in transferred cells, but no virus was detected either before or after ConA stimulation in spleen or lymph node cells. Different numbers of cells were inoculated into mice, and 2 x 10^7 cells/mouse were found to be required to induce disease (Table 22). None of the mice in the group inoculated with 1 x 10^7 or 5 x 10^6 ConA activated spleen cells developed disease.

Induction of EAE in Naive Mice with Cells Prepared from BALB/c Mice Treated with Both Neuroantigen and Virus and Stimulated with Myelin Basic Protein in vitro

If MBP is the encephalitogen in this model, incubation of lymph node or spleen cells with MBP may specifically stimulate the effector cell population and therefore may

Table 21: Induction of EAE in Naive Mice with Spleen Cells(Sp.) or Lymph Node Cells(LN) from BALB/c Mice Treated with Different Regimens

Group	Cells[a] transferred	EAE Induction[b]	EAE mice/ Total Transferred Mice[c]	Percentage of EAE Mice (%)
1	Sp [MSCH+A7][b]	+	8/40[d]	20
2	Sp [MSCH]	-	0/20	0
3	Sp [A7]	-	0/20	0
4	LN [MSCH+A7]	+	2/10[e]	20
5	LN [MSCH]	-	0/10	0
6	LN [A7]	-	0/8	0

a: Cells were prepared at the time 10 or 12 days after MSCH inoculation and/or 3 days after virus infection. b: MSCH: mouse spinal cord homoganate; A7: strain A7 of Semliki forest virus. b: Mice were irradiated with 500 R one or two hr before transfer. Pertussis vaccine was injected i. v. at days 1 and 3 after transfer. c: Accumulated data from four separate experiments. d: significant from group 2 and 3, $p < 0.05$. e: significant from group 5 and 6, $p < 0.05$.

Table 22: Effect of Cell Number on Induction of EAE in Naive Mice with Spleen Cells from BALB/c Mice Treated with Different Regimens

Cells from[a]	Cells /mouse	EAE induction	EAE mice/ recipients[c]	EAE onset (%)	Day EAE onset	Neuronologic signs
I[b] (MSCH + A7)	$3x10^7$	+	2/10	20	12	++ to +++
	$2x10^7$	+	2/10	20	12	++
	$1x10^7$	-	0/10	0	-	-
	$5x10^6$	-	0/10	0	-	-
II (MSCH)	$3x10^7$	-	0/10	0	-	-
	$2x10^7$	-	0/10	0	-	-
	$1x10^7$	-	0/10	0	-	-
	$5x10^6$	-	0/10	0	-	-
III (A7)	$3x10^7$	-	0/10	0	-	-
	$2x10^7$	-	0/10	0	-	-
	$1x10^7$	-	0/10	0	-	-
	$5x10^6$	-	0/10	0	-	-

a: Cells were preparated at day 10 p.i. MSCH inoculation and/or day 3 p.i. A7 infection.
b: MSCH: mouse spinal cord homogenate. A7: strain A7 of SFV. All animals were inoculated with Pertussis vaccine as well.

increase the rate of disease onset. To test this, lymph node cells obtained from mice inoculated with neuroantigen and virus at day 10 after inoculation of neuroantigen were incubated in vitro with MBP (50 μg/ml) for 4 days, and 2 x 10^7 viable cells were transfered to BALB/c mice with the same procedure as ConA activated cells. However, only 3 of 10 mice which received MBP stimulated cells developed neurologic signs (2+) at day 12 - 13 after inoculation of cells. Therefore, even though MBP stimulated cells can also transfer the disease, the cells did not differ from ConA activated lymph node cells in terms of rate of disease onset. This experiment indicates that MBP plays a role in induction of EAE in BALB/c model.

FACS analysis was used to reveal the phenotypes of lymph node cells before and after MBP stimulation *in vitro*. The cell with L3T4 phenotype increased from 35.5 to 72.8%, and Ia antigen positive cells increased from 12.4 to 36.9%. This was in agreement with the general notion that L3T4+ T cells may have a major role in inducing EAE.

Chapter Five

Studies on the Possible Mechanisms of SFV-induced Potentiation of EAE Development

IFN Production by Immune Cells from BALB/c Mice Infected with A7-SFV

As the potentiation effect of A7-SFV is at least partially immunologically mediated, it is necessary to analyse the possible immunological changes in mice caused by SFV infection. As IFN is an immunological modulator (214), the production of IFN after A7-SFV infection in BALB/c mice was investigated. Groups of BALB/c mice were inoculated with 10^6 PFU of A7-SFV intraperitoneally. At different days after virus infection, spleen cells, lymph node cells and peritoneal cells were pooled from five mice. These cells were incubated *in vitro* in the presence or absence of an optimal ConA concentration (2.5 μg/ml) and supernatants were harvested by centrifugation and UV inactivated. No infectious virus was found in the UV inactivated supernatants. The level of IFN was measured by a conventional biological assay.

A small amount of IFN was detected in lymph node, spleen and peritoneal cell supernatants prepared from mock-infected mice, but greater amount of IFN were

produced by lymph node and peritoneal cells from infected mice at day 1 to 5 after infection (Table 23). The largest increase of IFN production was found in peritoneal cells from virus infected mice at day 1 and 2 after virus infection. Small amounts of IFN were produced by spleen cells from mock infected mice, and no difference was observed between spleen cells from mock infected mice and from virus infected mice at observed times after virus infection (Table 23). Spontaneous production of IFN was found in peritoneal cells at days 3 and 4 after A7-SFV infection, day 4 in spleen cells. Spontaneous production of IFN is evidently a short-term event, as it was not detected at other times.

Since both IFNα,β and IFNγ can protect L929 cells from the cytopathic effect of vesicular stomatitis virus (VSV), further efforts were taken to differentiate between IFNα,β and IFNγ. Anti-IFNγ was added together with the supernatant (Table 23) to L929 cell monolayers. In spleen or lymph node cell cultures from mock infected mice, only IFNγ was produced under the stimulation of ConA as the anti-IFNγ MAb did completely neutralize the activity. The IFNs activity of the culture supernatant of spleen or lymph node cells from virus infected mice was also completely neutralized by anti-IFNγ MAb, but not the IFN activity of culture supernatants of peritoneal cells. It was concluded that IFNγ was the principal IFN type produced by spleen and lymph node cells, and IFNα,β was predominantly released by

Table 23: IFN Production in BALB/c Mice after A7-SFV Infection *in vivo*[a]

Day p.i.	IFN (U/ml)[b] produced from					
	Spleen cells		Lymph node cells		Peritoneal cells	
	Sti.[d]	Spo.[e]	Sti.	Spo.	Sti.	Spo.
mock infected	22	-[c]	40	-	40	-
1	17	-	80	-	160	-
2	17	-	100	-	160	-
3	34	-	56	-	124	80
4	34	40	64	-	124	80
5	40	-	64	-	80	-
6	17	-	40	-	40	-
7	17	-	40	-	40	-

a: Cells were pooled from five mice in each group. The daily variation of IFN production in the mock infected group was within 10%. b: In this experiment, both 4.3 IU/ml IFN-γ and 10 IU/ml IFN-α,β protected against VSV CPE. The results in the Table are expressed as U/ml using recombinant IFNγ as a standard. c: not detected. d: Stimulated by ConA. e: Spontaneous production.

peritoneal cells after SFV infection.

Production of IL-2 in BALB/c Mice After A7-SFV Infection In vivo

Supernatants of ConA stimulated cell cultures from mice infected or mock infected with A7-SFV were tested for the presence of IL-2 with IL-2 dependent CTL line. An increase in production of IL-2 was found in spleen cells from virus infected mice day 3 after virus infection (Table 24). Elevated levels of IL-2 was also detected in supernatants harvested from lymph node cells from virus infected mice. Spontaneous production of IL-2 was not detected in the supernatants of different cell populations. It can be concluded that production of IL-2 in BALB/c mice occurred during virus infection, but the amount produced was dependent on different cell population.

Transiently Induction of IL-1 Production in A7-SFV Infected BALB/c Mice In vivo

An increased production of IL-1 was found in lymph node cells from mice infected with A7-SFV either at day 3 or day 4 after infection. No significant difference was observed between supernatants harvested from spleen cells of mock infected or virus infected mice. The level of IL-1 in supernatants of peritoneal cells from virus infected mice was lower than that of mock infected mice (Table 25). It was assumed that the effect of A7-SFV infection on IL-1

Table 24: Production of IL-2 in BALB/c Mice after A7-SFV Infection *in vivo* [a,b]

Day p.i.	IL-2 from		
	Spleen cells	Lymph node cells	Peritoneal cells
mock-infected	20436 ± 2012	14250 ± 1672	19264 ± 2047
1	19388 ± 946	20648 ± 482	9388 ± 1020
2	24076 ± 1924	20420 ± 1207	25348 ± 1762
3	45160 ± 3426	28152 ± 2042	24112 ± 1684
4	36480 ± 3247	35328 ± 3042	21060 ± 2004
5	24740 ± 1862	24864 ± 1724	22536 ± 1424
6	28476 ± 3010	8974 ± 872	18401 ± 1762
7	10436 ± 107	4984 ± 362	14192 ± 1024

a: Cells were pooled from five mice in each group. The daily variation of IL-2 production in mock infected group was within 10%.b: The measurment of IL-2 production was done according to Gearing et al, 1985. The ability of the supernatants to support proliferation of IL-2 dependent CTL line cells was measured by [^{3}H]-thymidine uptake during the last 18 h of the 48 h incubation time (cpm/min).
c: The results were expressed as the mean ± standard deviation of the incorporated [^{3}H]-thymidine in quadraplicate cultures. A representative experiment of three separate experiments is shown.

Table 25: Production of IL-1 in BALB/c Mice after A7-SFV Infection *in vivo*

Day p.i.	IL-1 Produced [a,b] from		
	Spleen cells	Lymph node cells	Peritoneal cells
	cpm[c]	cpm	cpm
Mock-infecetd	33883 ±1126	6629 ±944	20846 ±2135
3	23334 ±1410	16641 ±1383	14262 ±883
4	37567 ±1327	15074 ±1820	8765 ±1231

a: Cells were pooled from five mice in each group and incubated in the presence of 2.5 µg/ml of ConA for 24 h. Supernatants were harvested and residual ConA was neutralized by adding β-methylmannoside (1 g/50 ml supernatant).

b: The procedure of IL-1 production was done according to Gearing et al, 1985. The ability of samples to support proliferation of thymocytes was measured by uptake of [^{3}H]-thymidine during the last 18 h of the 48 h incubation time.

c: The results were expressed as the mean ± standard deviation of the incorporated [^{3}H]-thymidine in triplicate cultures.

production could also be a time dependent phenomenon as observed in IL-2 and IFNγ productions. To search this possibility, peritoneal cells were prepared from virus or mock infected mice at days 1, 2, 3, 4 p. i., and supernatants were tested for the level of IL-1. A transient IL-1 production was induced by virus infection at days 1 and 2 p. i. Afterwards, the level of IL-1 declined, and at day 4, to the level which was lower than that of mock infected control (Table 26).

Effect of Systemic Administration of IFNγ on EAE Development

Since virus infection stimulated IFNs production *in vivo*, it could be logical to ask whether recombinant IFNγ can replace virus infection and potentiate the disease. 10,000 IU of recombinant IFNγ were inoculated i.v. into each mouse at days 9, 10 and 11 post neuroantigen inoculation. Control mice were infected or mock infected with A7-SFV at day 7 post neuroantigen inoculation. Administration of IFNγ *in vivo* did not potentiate disease (Table 27). In mock infected mice, about 40% mice developed disease at day 24 post neuroantigen inoculation. In the IFNγ administered group, only 13 - 20% of mice developed EAE at day 24 post neuroantigen inoculation. It is apparent that IFNγ administration *in vivo* could not replace the virus infection to potentiate the EAE development in BALB/c mice.

Table 26. IL-1 Production by Peritoneal Cells after A7-SFV Infection *in vivo*[a,b]

Day p.i.	IL-1 Produced (cpm/min)	
	Ex. 1	Ex. 2
Mock-infecetd	18780 ± 2142	21427 ± 1926
1	34852 ± 3729	38720 ± 4012
2	20637 ± 1982	24072 ± 2001
3	15460 ± 1784	17043 ± 1602
4	10000 ± 1216	14014 ± 1203

a: Cells were pooled from five mice in each group. The results were expressed as the mean ± S.D. of quatruplica cultures.
b: The experimental procedures were the same as described in Table 22.

Table 27: Effect of Systemic Administration of IFNγ on EAE Development

group	Treatment of micea (p.i. MSCH)		EAE mice/ Total mice	Disease Onset (%)
	IFNγ	Virus		
1	+	-	4/25[c]	16
2	-	+	17/20	85
3	-	-	8/20[d]	40

a: Mouse spinal cord homogenate.
b: Amount of 10,000 I.U./mouse was inoculated i.v. at day 9,10,11 post MSCH inoculation.
c: significantly different from group 2, $p < 0.001$. Not significantly different from group 3, $p < 0.1$. d: significantly different from group 2, $p < 0.01$.

Effect of Cell Transfer from A7-SFV Infected Animals on EAE Development in Recipient BALB/c Mice

Instead of inoculation of A7-SFV at day 7 to neuroantigen primed mice, 5 x 10^7 spleen cells from virus infected mice at day 5 p.i. was inoculated into each mouse i.v. to see whether these virus-primed cells could also potentiate EAE. To investigate virus presence, spleen cells were cultivated *in vitro* on the MBA-13 cell line (infectious center assay). Spleen cells were also cultured in the presence of ConA, supernatants harvested and tested for infectivity. No infectious virus was detected.

About 45% of the mice which received both neuroantigen and virus primed spleen cells developed EAE at day 17 after neuroantigen inoculation as compared to mock inoculated mice of which 30% developed disease at day 24 - 26 after neuroantigen inoculation (Table 28). Lymph node cells harvested at day 5 after infection were also able to potentiate the disease. In a separate experiment, 5 of 10 neuroantigen primed mice developed EAE at day 15 - 17 after receiving lymph node cells. Therefore, virus primed spleen or lymph node cells seem to potentiate EAE development possibly by increasing the incidence but especially by shortening the time of disease onset.

Effect of Subpopulation Depletion on EAE Potentiation by Transferred Cells

To investigate which cell population participated in the

Table 28: Effect of Virus Primed Spleen Cells on EAE Development

Group	Treatment of Mice at Day 7 p.i. MSCH[a] Inoculation			EAE mice /Total mice	Disease Onset(%)	Time of Disease Onset (day)
	Primed Spleen Cells[b]	Normal Spleen Cells	A7-SFV Infection			
1	+	-	-	9/20[c]	45	17
2	-	+	-	3/10[e]	30	25
3	-	-	+	8/10	80	14
4	-	-	-	3/10	30	25
5	-	-	-[d]	3/10	30	25

a: Mouse spinal cord homogenate.
b: Total of $5x10^7$ spleen cells from virus infected mice at day 5 p.i. was inoculated per mouse i.v.. No infectious virus was detected in the supernatant of cell culture or cells with microtitration assay or infectious center assay.
c: Pooled data from two separate experiments. Not significant difference, compared to group 2, 4, and 5, $p<0.3$ respectively.
d. Donor spleen cells were incubated in vitro in the presence of ConA (2.5ug/ml) at the $3x10^6$ cells/ml, supernatant was harvested, U.V. irradiated and 0.2 ml was inoculated into each recipient mouse at day 7 after neuroantigen inoculation.
e: Not significantly different, compared to group 1, $p< 0.3$.

potentiation effect, spleen cells from A7-SFV infected mice were depleted of different cell populations and injected into neuroantigen primed mice. B cells were depleted by a T cell enrichment column. The efferent cells from T cell column contained 92 - 95% Thy. 1 positive cells, and 5 - 7% Mac-1+ cells. Depletion of B cells did not effect the potentiation (Table 29). Four of nine neuroantigen primed mice developed EAE after receiving B cell depleted spleen cells similarly as mice receiving unfractinated spleen cells from A7 infected mice. However, depletion of macrophages by two cycles of adherence to plastic plates abolished the potentiatic effect, as none of the mice developed EAE after receiving macrophage depleted spleen cells. To investigate whether only spleen macrophages (radio-resistant cells) can potentiate disease, spleen cells were irradiated with 1500 R, and injected into neuroantigen primed mice. An irradiation dose of 1500 R was also shown to completely abolish the proliferation ability of spleen cells in response to mitogen (ConA). Only one mouse developed typical paralysis after receiving 1500 R irradiated spleen cells from A7 infected mice. This was not related to materials released by dead cells after irradiation, since normal spleen cells irradiated with 1500 R did not have the same effect. Furthermore, no potentiation effect was found with normal spleen cells.

Transient Induction of IL-1 on Peritoneal Macrophages (PM)

by SFV Infection In vitro

SFV infection alone induced IL-1 production in resting peritoneal macrophages, as did both the W-SFV and A7-SFV (Fig. 9, 10). The release of IL-1 from virus infected macrophages was a time and infectious dose dependent event. The maximal production of IL-1 by infected macrophages was at 8 h after infection, and macrophages infected at a m.o.i. of 10 produced the highest level of IL-1. Afterwards, the IL-1 production gradually declined. IL-1 production by stimulation through adherence to plastic plates (177) can be ruled out as the macrophages had been incubated *in vitro* for 48 h, and IL-1 could not be detected before infection. Mock infected macrophages also produced small amount of IL-1 after 24 h. This small amount of IL-1 may be derived from the stimulation of macrophages by 2-mercaptoethanol and serum components in fresh medium. As it has been earlier demonstrated, 2-mercaptoethanol has a stimulating effect that induces IL-1 production by resident macrophages (178, 179, 180). Stimulation by 2-mercaptoethanol and serum components may also be a transient event, as there was no IL-1 production 48 h after mock infection. Since indomethacin, an inhibitor of the cyclooxygenase pathway of macrophage metabolism, was constantly present in culture medium, the involvement of prostaglandin E series as a mediator can be excluded (181 - 183).

Table 29: Effect of Depletion of Different Cell populations on EAE Potentiattion by Spleen Cells from A7-SFV Infected Mice[a]

gp.	Normal spleen cells	In vitro Treatment of spleen cells from A7 infected mice			EAE mice/ Total mice	Disease onset (%)	Day of disease onset[b]	Neurologic signs
		passing T cell column	Plastic adherence	1500R				
1	+	-	-	-	2/8c	25	24	++
2	-	-	-	-	5/8	62	13 - 14	++ to +++
3	-	+	-	-	5/8	62	13 - 14	++ to +++
4	-	-	+	-	0/8d	-	-	-
5	-	-	-	+	1/7e	14	12	++

a. Spleen cells were prepared from mice infected or mock infected with A7-SFV 4-5 days earlier. After lysis of red blood cells the spleen cells were washed 3x with PBS and fractionated with T cell enrichment column, plasitic adherence or irradiation. Group 1 receivd $6x10^7$ normal spleen cells/mouse; Group 2 received $6x10^7$ cells/mouse from A7-SFV infected mice; Group 3 received $2x10^7$ cells from T enrichment column; Group 4 received $2x10^7$ adherent cells and group 5 received $6x10^7$ irradiated cells/mouse. b. Day after neuroantigen inoculation. c. Significantly different from groups 2 and 3, $p<0.05$ respectively. d. Not significantly different from group 1. e. Not significantly different from group 1.

Figure 9 and 10. Transient induction of interleukin-1 production on resting macrophages by SFV. The amount of IL-1 in the supernatant harvested from each culture was determined by the ability of samples to support [^{3}H]-methylthymidine uptake of murine thymocytes with the stimulation of suboptimal concentration of ConA (1.25 μg/ml). Indomethacin (10μM/ml) was present during the entire incubation period. The background in this representative experiment was as following: ConA + Thymocytes= 2684 ± 107. Medium= 454 ± 10. W-10: Cells infected at m. o. i. of 10 with W-SFV. W-1: Cells infected at m. o. i. of 1 with W-SFV. W-0.1: Cells infected at m. o. i. of 0.1 with W-SFV. A7-10: Cells infected at m. o. i. of 10 with A7-SFV. A7-1: Cells infected at m. o. i. of 1 with A7-SFV. A7-0.1: Cells infected at m. o. i. of 1 with A7-SFV.

Figure 9

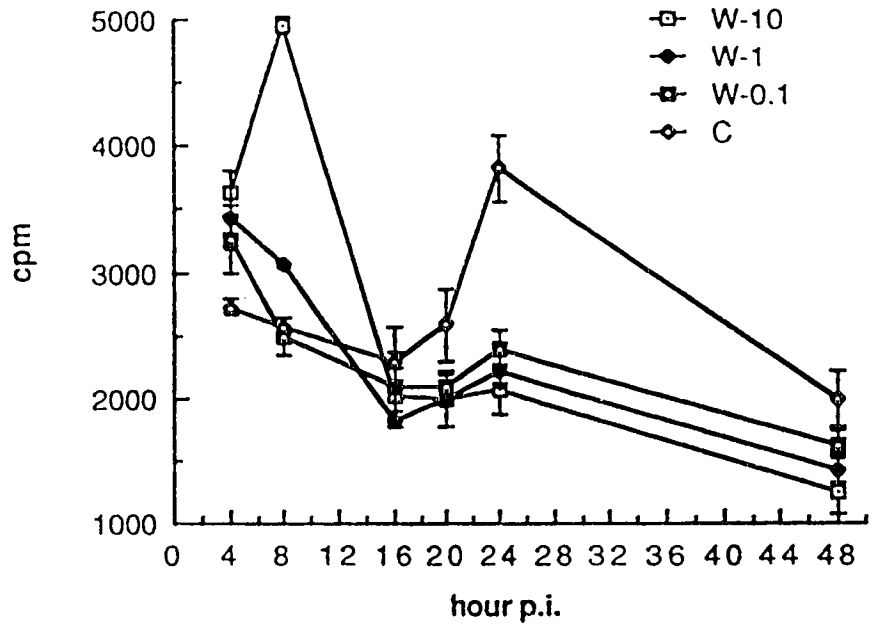
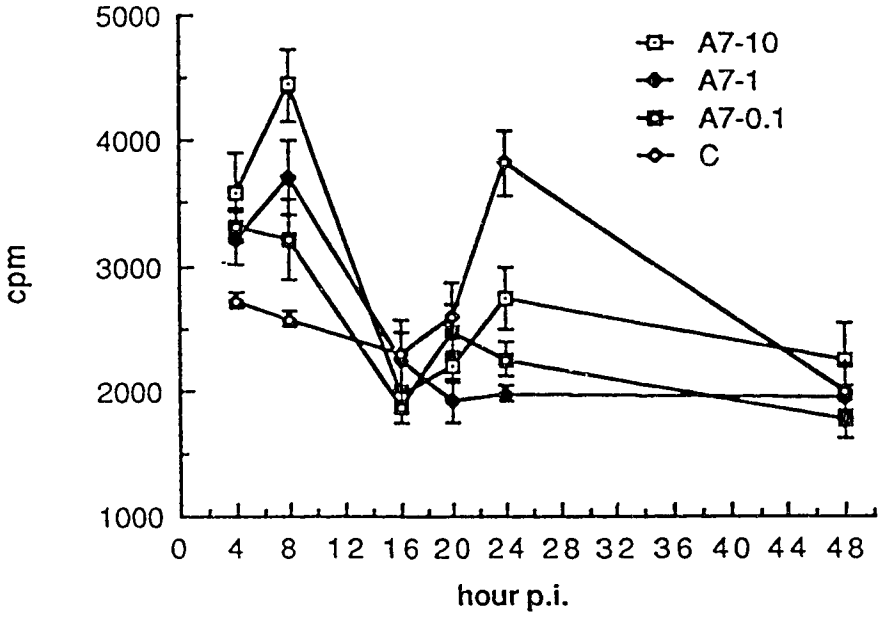


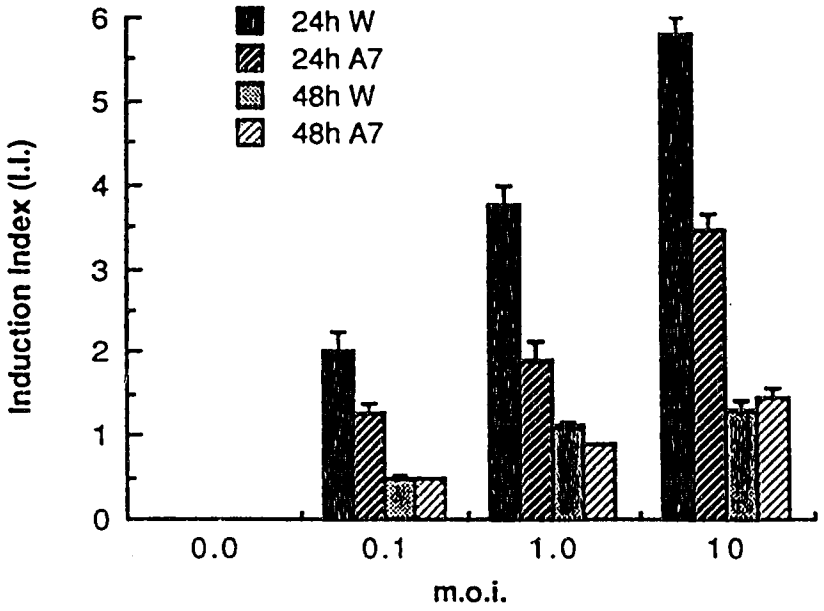
Figure 10



Induction of Ia Antigen Expression on PM by SFV Infection

It has been demonstrated that both synthesis and expression of Ia antigen in peritoneal macrophages are transient events (184). In a time dependent manner, the number of Ia antigen positive cells decrease dramatically 16 h after seeding onto plates (184). Twenty four h after removal from the peritoneal cavity, the Ia antigen expression of the PMs is almost undectable. However, re-expression can be induced by phagocytic stimuli (184). It was also observed in my experiments that 48 h after seeding onto 24 well plastic plates, the Ia antigen positive cell number in PMs was about 2 -5% (FACS analysis). Rat ConA supernatant induced expression of Ia antigen. The percentage of Ia antigen positive cells increased in a time dependent manner, eg., 20 - 30% after 24 h incubation time, 50 - 60% after 48 h. It was observed that virus infection alone can induce Ia antigen expression on resting PM. Two important aspects of this induction must be emphasized (Fig. 11). First, induction of Ia antigen expression by virus infection was a transient event. The Ia antigen expression on infected PM reached the peak level 24 h after infection, and decreased afterwards. This phenomenon occurred with both W-SFV and A7-SFV infected PM. Second, the induction of Ia antigen expression by virus infection was dose dependent, namely, more Ia antigen positive cells appeared in PM populations infected by a high multiplicity of infection (m. o. i. of 10). However the Ia antigen expression in macrophages

Figure 11. Semliki Forest virus infection of resting macrophages transiently induces Ia antigen expression. One million 1% formaldehyde fixed cells were analysed by FACS at an excitement wave length of 488 nm and expressed as cell number/log scale of fluorescence intensity. The results are shown as Induction Index (I.I.) = (% virus infected cells - % noninfected cell)/% noninfected cell. Data from 3 experiments expressed as Mean±S. D. W-10: Cells infected at m. o. i. of 10 with W-SFV. W-1: Cells infected at m. o. i. of 1 with W-SFV. W-0.1: Cells infected at m. o. i. of 0.1 with W-SFV. A7-10: Cells infected at m. o. i. of 10 with A7-SFV. A7-1: Cells infected at m. o. i. of 1 with A7-SFV. A7-0.1: Cells infected at m. o. i. of 1 with A7-SFV.


24h W
24h A7
48h W
48h A7
Induction Index (I.I.)
0
1
2
3
4
5
6
0.0
0.1
1.0
10
m.o.i.

infected with a low m. o. i. of 1.0 or 0.1 was equivalent to the uninfected control.

It is known that SFV induce IFNγ production in murine spleen cells (185). To help understand the mechanism of induction of Ia antigen expression on PM by SFV infection, I further investigated whether induction of Ia antigen expression was mediated by IFN produced by PM after virus infection. The supernatant from infected PMs was harvested at 24 h p.i.. After U. V. inactivation of virus, IFN were titrated on L929 cells as described in Materials and Methods. Both IFNα,β and IFNγ standards protected L929 cells from the cytopathic effect of VSV but none of the supernatants did. It seems, therefore, that IFN did not play a major role in this experimental system even though endogeous IFN may exist. UV inactivated virus was also used to investigate the induction effect at the same m.o.i., but only an insignificant effect was observed, even at a m.o.i. of 10.

Other viruses were also chosen as control to observe the Ia induction effect (Table 30). As measles virus (MV) can cause also an abortive type infection in murine macrophages *in vitro* and different strains may cause different type of infection (243, 246), these MV strains were included. All three strains induced Ia antigen expression but U V inactivated measles virus did not have any significant effect. The results were similar as in A7-SFV infected cells. Herpes simplex virus-1 infected PM and

Table 30: Effect of Virus Infection on the Expression of Ia Antigen on Murine Peritoneal Macrophages (PM)[a]

Virus (strain)	Viral antigen[b] synthesis in PM	Induction of Ia Antigen on Resting PM
Semliki forest virus (SFV)		
W-SFV	+	+
A7-SFV	+	+
UV-inactivated		
W-SFV	+	-[b]
A7-SFV	+	-[b]
Measles Virus		
Lec	+	+
Ts[c]	+	+
Halle	+	+
UV-inactivated		
Lec	+	-[b]
Ts	+	-[b]
Hale	+	-[b]
Purified virion (Lec)	+	+
Herpes simplex virus-1	+	+
Human adenovirus Type-3	-	-
Poliovirus-I	-	-

a: thioglycollate elicited peritoneal macropghages b: presence of intracellular viral antigen was determined by immunofluorescence after infection c: 37C is a nonpermissive temperature. b: insignificant.

induced high level Ia antigen expression (Fig. 12). In contrast, both human adenovirus-3 and poliovirus could not infect PMs and did not induce Ia antigen expression.

Effect of SFV Infection In vivo on the Antigen Presenting Ability of Murine Spleen Macrophages

It is known that the antigen presenting ability of different macrophage population is correlated with the amount of Ia antigens expressed on the macrophage surface (187). Therefore the induction of Ia antigen expression on PM by virus infection may influence the ability of antigen presention. To examine this possibility, mouse spleen cells were harvested from A7 infected or mock-infected mice at different times after infection, and irradiated with 1500 R, a dose shown to be lethal for T and B cells but harmless to macrophages. Irradiated spleen cells were used as antigen presenting cells for OVA-specific primary T cell line cells. Prelimary experiment demonstrated that a T cell : Irradiated spleen cell ratio of 2 : 10 had an optimal efficiency. Antigen presenting cells from A7-SFV infected mice at day 1 or 2 after virus infection had a much higher efficiency (50% increase) in antigen presenting ability as compared with normal spleen cells. Fig. 13 shows a representative experiment. It is clear that proliferation of OVA-specific T cells was caused by interaction between T cells, antigen presenting cells and antigens, since in the case of only T cells and macrophages or T cells and

Figure 12. FACS analysis of Ia antigen expression on PM after virus infection. 1. Infected with A7-SFV at m. o. i. of 100 and stained with normal rat IgG; 2. Mock-infected PM; 3. Infected with A7-SFV at m. o. i. of 100; 4. Infected with A7-SFV at m. o. i. of 10; 5. Infected with MV (strain Lec) at m. o. i. of 10; 6. Infected with MV (strain Lec) at m. o. i. of 1; 7. Infected with HSV-1 at m. o. i. of 10; 8. Infected with HSV-1 at m. o. i. of 1; 9. Infected with Human adenovirus-5; 10. Infected with poliovirus-1; 11. Mock-infected PM; 12. Infected with MV at m. o. i. of 10 and stained with normal rat IgG.

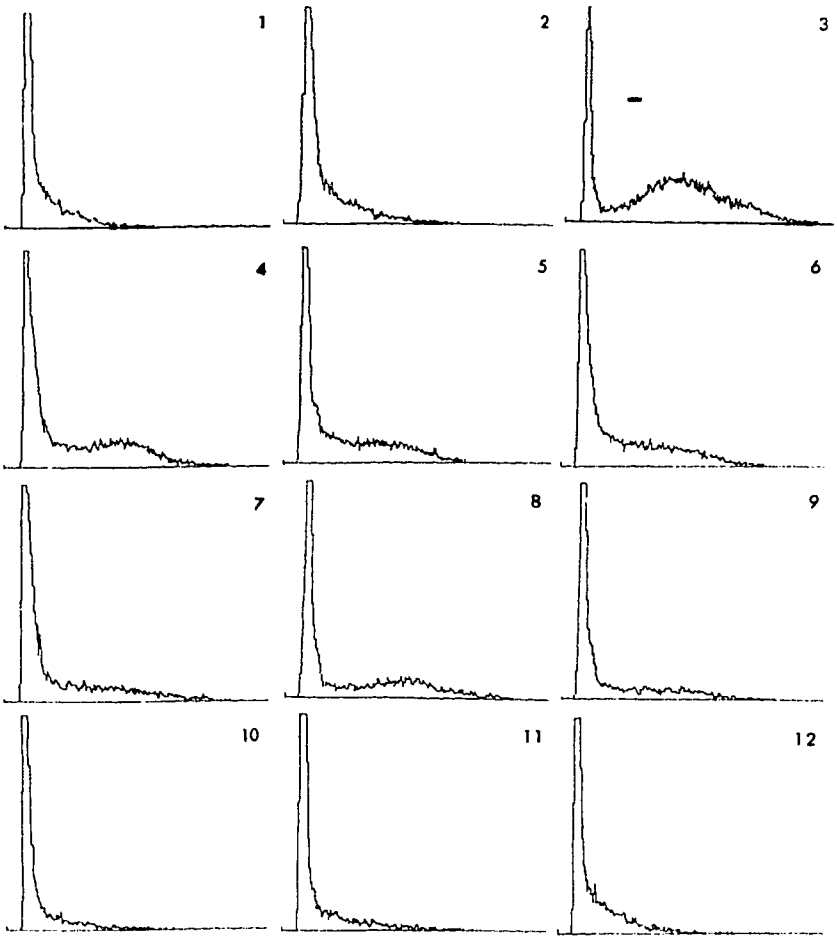
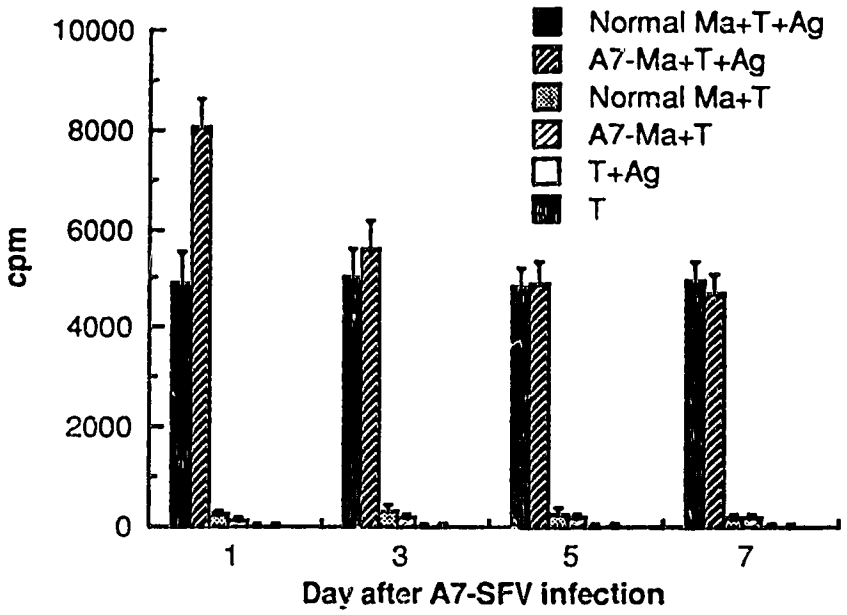

1
2
3
4
5
6
7
8
9
10
11
12

Figure 13. SFV infection *in vivo* temporaly upregulates the antigen presenting ability of murine spleen macrophages. Spleen cells were prepared from A7 or mock-infected mice at different times after infection. Cells were pooled from five mice at each day in each experiment. Spleen cells free of red blood cells were irradiated with 1500 R and then used as antigen presenting cells. OVA specific T cells were prepared from mice immunized with OVA (20 μg/mouse in CFA) 9-10 days before cells were taken and generated as described in *Materials and Methods.* $2X10^4$ T cells/well and $1X10^5$ irradiated spleen cells were used in this representative experiment. Cells were pulsed with 0.4 μci/well of [^{3}H]-thymidine at the last 18 h of 96 h incubation period and expressed as mean±S. D. Normal Ma: Irradiated spleen cells from mock-infected mice. A7-Ma: Irradiated spleen cells from A7 infected mice. T: OVA specific T cells. Ag: OVA.

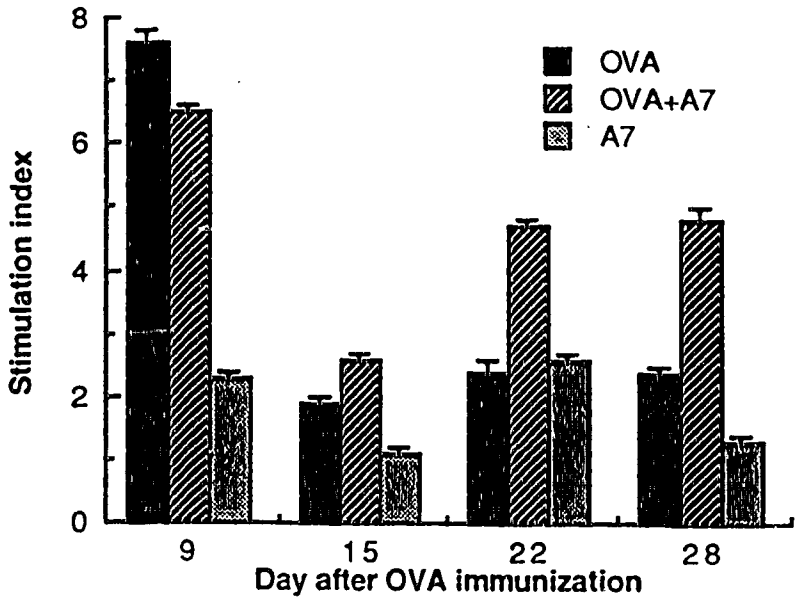

Normal Ma+T+Ag
A7-Ma+T+Ag
Normal Ma+T
A7-Ma+T
T+Ag
T
cpm
10000
8000
6000
4000
2000
0
1
3
5
7
Day after A7-SFV infection

antigens, no proliferation was found. Spleen cells from A7-SFV infected mice at day 3 after infection had a slightly higher or equal efficiency in antigen presenting as compared with normal spleen cells (Fig. 13). Afterwards, until day 7 after virus infection, the antigen presenting ability of spleen macrophages from either A7 or mock-infected mice were not different. Therefore, upregulated antigen presenting ability by virus infection was a time dependent event and found only early after virus infection.

Effect of A7-SFV Infection in BALB/c Mice in vivo on the T Cell Reactivity to Ovalbumin (OVA).

A7-SFV infection had an effect in enhancing autoreactivity to neuroantigen (MSCH) in a time dependent manner *in vivo*. The phenomenon may be a neuroantigen specific effect, especially since SFV is a neurotropic virus, but it may also be more general. To investigate this question, A7-SFV was injected into control mice and immunized with OVA. Lymph node cells were prepared from mice treated with different regimens at different times after infection, and stimulated by OVA *in vitro*. At day 9 after OVA immunization, no significant difference could be found between lymph node cells from A7 infected or mock-infected mice (Fig. 14). At day 15, the responses to OVA declined in both groups. Nevertheless, a significantly higher level (50% increase) of proliferation was found with lymph node cells from OVA immunized and virus infected mice at day 22

Figure 14. Effect of A7 infection in BALB/c mice in vivo on T cell reactivity to immunogen (OVA). Experimental procedure was described in *Materials and Methods*. Mean±S.D. of stimulation index (S.I.= cpm of cells stimulated by antigen/ cpm from cells without antigen stimulation) from three separate experiments is shown.


8
6
4
2
0
Stimulation index
OVA
OVA+A7
A7
9
15
22
28
Day after OVA immunization

and 28 after OVA immunization. In contrast, the proliferation of lymph node cells from OVA immunized mice remained at base line level. No substantial differences in proliferation to SFV antigen were found between mice infected with A7-SFV and mice immunized and infected with A7-SFV.

These results of the effect of A7-SFV infection on the OVA specific cellular response are in line with the observed changes in the EAE model and show that an unrelated immune response can be modulated by A7-SFV infection. They also confirm that the effect of virus infection depends on the time after immunization.

DISCUSSION

This thesis project was intended to establish a mouse EAE model which could be used to observe the modulating effect of virus infection on the onset and development of the disease. The model should be able to provide useful information which cannot feasibly be obtained in clinical studies,e.g., the possible mechanism of an exacerbating effect of virus infection on MS relapse. It was demonstrated that EAE can be induced in genetically resistant BALB/c mice with the aid of low dose irradiation. A7-SFV infection enhanced the rate and shorten the time of disease onset. This suggests that the potentiation effect was mediated immunologically. A7-SFV infection did not delete immune cells which are important for the disease induction and development. Instead, A7-SFV infection in BALB/c mice primed with neuroantigen disturbed the immunological network by inducing cytokine production and/or redistribution of immune cells, and transiently activated macrophages and certain subset(s) of T cells. These activated macrophages may behave as effective autoantigen presenting cells in co-operating with a subset of T cells, further activating a pre-existing but suppressed T effector cells of EAE and, therefore, induce and/or exacerbate the disease.

Based on this study, the following facts can be stated:

1. EAE can be induced in the genetically resistant BALB/c mouse strain with the aid of low dose irradiation. 350R irradiation two days prior to neuroantigen inoculation was the optimal dosage (Chapter 1, Table 1).
2. Two unique features were observed in BALB/c mice in contrast to SJL/J mice. First, the rate of disease onset was much lower in BALB/c mice than in SJL/J mice, the former being 20-30%, and the latter about 80%. Second, the time of disease onset was later in BALB/c mice than in SJL/J mice, the former being 24 days on the average, the latter being 11-13 days (Chapter 1, Table 2).
3. These two unique features of the BALB/c mouse model are advantageous for observing the modulation effect of virus infection on EAE development, in particularly the potentiation effect. An avirulent strain of SFV, A7-SFV, was introduced into this model. Intraperitoneal A7-SFV injection not only enhanced the rate of disease onset from 20-30% to 70-80%, but also shortened the time of disease onset from 24 days to 12-14 days. With low dose irradiation and virus infection only, only about 10% mice developed paresis at day 8-9 after infection which was distinct from mice treated with 350R, neuroantigen and virus infection. Low dose irradiation, neuroantigen inoculation and A7-SFV infection were all important

elements in this model (Chapter II, Table 3).

4. The potentiation effect of A7-SFV infection was time-dependent. A7-SFV infection at day 7 after neuroantigen inoculation had the highest effect, but at day 3, A7-SFV infection had no potentiation effect (Chapter II, Table 4).

5. The potentiation effect of A7-SFV infection was dose-dependent. 10^6 PFU of A7-SFV per mouse had the highest potentiation effect. 10^2 or 10^8 PFU per mouse did not enhance the rate of disease onset, but did shorten the time of disease onset (Chapter II, Table 5).

6. No potentiation effect was observed with inactivated virus (Chapter II, Table 6).

7. Preceeding A7-SFV infection at 21 days before neuroantigen inocubation did not have a potentiation effect in BALB/c mice (Chapter II, Table 9).

8. A7-SFV infection in vivo augmented reactivity to mitogen in neuroantigen inoculated mice (Chapter II, Table 10).

9. Lymphocyte transformation assays suggested that neither BP nor PLP was major encephalitogen in this model (Chapter II, Table 10).

10. Variation of cellular composition was found in both spleen and lymph node in mice treated with different regimens, e. g., $L3T4^+$ T cells in lymph node were increased at day 9 after neuroantigen inoculation in mice treated with neuroantigen and A7-SFV, but decreased significantly in mice treated with neuroantigen alone. A7-

SFV infection alone also increased L3T4$^+$ T cells in lymph node. Even though no general rule can be generated from this investigation this data suggested that a general change in immunological response was involved in the potentiation effect (Chapter II, Table 11).

11. Anti-L3T4, anti-Lyt.2, F4/80 and control antibody (normal rat IgG) was administered into mice which received neuroantigen and virus infection. Both anti-L3T4 and F4/80 antibodies reduced dramatically the disease onset, confirming the role of L3T4+ and T cells and macrophages in this model. About 20-30% mice died after *in vivo* administration of antibodies, which was also observed in mice infected with virus only, therefore, *in vivo* administration of anti-L3T4, or anti-Lyt.2, or F4/80 may inhibit cells responsible for restricting virus infection, which leads to an increased virulence of A7-SFV. Nevertheless the data suggested that the effector cells in this model are the same as in other EAE models (Chapter II, Table 12).

12. Replication kinetics of A7-SFV in BALB/c mice were investigated *in vivo*. In both BALB/c and SJL/J strains, A7-SFV caused a transient viremia. At day 4 after infection, no infectious virus was detected in blood (Chapter III, Table 13).

13. Infectious virus production in BALB/c mouse brains was also investigated. Virus replication peaked at day 5 after infection. At day 8 p.i., no infectious virus was detected

(Chapter III, Table 14).

14. Slot blot hybridization of SFV RNA with probes which recognize E1,and E2/E3 coding regions supported the above observation. At day 12 after A7-SFV infection, viral RNA was barely detectable (Chapter III, Figure 8).

15. Spleen mononuclear cells were infected with SFV *in vitro*, in order to investigate whether certain subpopulation of immune cells were preferentially infected or killed by virus infection. The data showed the Mac-1+ cells were the main cells infected by SFV (Chapter III, Table 17).

16. A7-SFV also caused a restricted infection of murine thioglycollate elicited peritoneal macrophages. Infectious virus was not detected at day 4 p.i., and viral RNA became undetectable at day 5 p.i. No cytopathic effect was found in infected macrophages. Both Ia^+ and Ia^- macrophages could be infected by A7-SFV. Therefore, it would appear that A7-SFV preferentially infected macrophages *in vitro* (Chapter III, Table 18, Figure 7).

17. Infection of bone marrow derived macrophages with SFV suggested that A7-SFV infection of macrophages could be related to the maturation stage but not activation stages of macrophages as mature macrophages were more susceptible to A7-SFV infection (Chapter III, Table 19, 20).

18. Spleen or lymph node cells were prepared from mice treated with different regimens at day 10 or day 12 after neuoantigen inoculation, incubated *in vitro* in the presence

of either Con A or BP, and then transferred to naive BALB/c mice which received 500 R irradiation 1 or 2 h before transfer. It was shown that only cells prepared from mice treated with both neuroantigen and virus infection can induce EAE in naive recipients. Infectious virus was not detected in these transferred cells with infectious center assay. About 20% of recipients developed paresis or paralysis at about day 12 after transfer. Even when the rate of disease onset was not high, the results suggested that the potentiation effect was immunologically mediated (Chapter IV, Table 21).

19. The effector cell number was also shown to be important in transferring the disease. Total of $2x10^7$ cells/mouse was a minimal requirement in tranferring the disease (Chapter IV, Table 22).

20. Virus infection may trigger the production of cytokines, i.e. IFN, IL-1, IL2 *etc.* These cytokines are important immunomodulators. Overproduction of cytokines may also induce or potentiate an autoimmune disease. In this model, the production of IFNs *in vivo* after A7-SFV infection induced a much higher level of IFN production than mock-infected control. At day 3 or 4 after infection, spontaneous IFN production reached peak level in spleen and peritoneal cells *in vivo* (Chapter V, Table 23).

21. IL-2 production *in vivo* after A7-SFV infection was also investigated. A7-SFV infection enhanced IL-2 production at day 3 or 4 after virus infection in both spleen and lymph

node cells (Chapter V, Table 24).

22. At day 1 after A7-SFV infection, IL-1 production was increased in infected mice. Afterwards, the production declined (Chapter V, Table 25).

23. Even though *in vivo* data demonstrated that A7-SFV infection can increase cytokine production, the contribution of this increased production of cytokine to the potentiation effect is not known. To directly assess the role of cytokine in potentiating the disease, recombinant IFN was administered in neuroantigen primed mice to replace A7-SFV infection. Three doses of 10,000 U per mouse were injected i.v. at day 9, 10, 11 after neuroantigen inoculation. However, no potentiation effect was observed. Thefore, even though increased production of IFN was observed, this may not be a major factor in this potentiation effect (Chapter V, Table 27).

24. To further study the immunological basis of the potentiation effect, spleen cells were prepared from A7-SFV infected mice and injected into neuroantigen primed mice at day 7 after neuroantigen inoculation. This approach did not significantly increase the rate of EAE. It did shorten, however, the time of disease onset from 24 days to 17 days after neuroantigen inoculation. Neither normal spleen cells nor ConA supernatant did have any effect. This effect was not mediated by infectious virus, as no virus was detected in transferred cells with an infectious center assay. This data indicated that this potentiation effect

was mediated by immune cells which were present in A7-SFV infected mice (Chapter V, Table 28).

25. To further analyse which subpopulations of immune cells participated in this potentiation effect, spleen cells prepared from A7-SFV infected mice were fractionated with different methods, and injected into neuroantigen primed mice. Depletion of B cells by T cell enrichment columns did not affect the potentiation effect. Nevertheless, two cycles of plastic adherence and 1500 R irradiation dramatically reduced the potentiation effect. This would suggest that both radiosensitive T cells and macrophages were important in this potentiation effect (Chapter V, Table 29).

26. The effect of A7-SFV infection on the functions and capacities of macrophages, and its relevance to the potentiation effect *in vivo* was also investigated in this project. A7-SFV infection of thioglycollate elicited peritoneal macrophages transiently induced IL-1 production *in vitro*. The production of IL-1 peaked at 8 hr. p.i. and decreased afterwards. This induction effect was also dose dependent, since a high m.o.i. had a more pronounced effect (Chapter V, Figure 9, 10).

27. A7-SFV infection also transiently induced Ia antigen expression in thioglycollate elicited peritoneal macrophages. The effect was pronounced at 24 h p.i. The Ia antigen induction effect was also observed with measles virus, and herpes simplex virus-1, but not with human polio

virus 1 and adenovirus 5. As measles virus and herpes simplex virus antigen synthesis can also be found in macrophages, the data suggested that this Ia antigen induction effect could be partially mediated by a receptor - ligand like interaction (Chapter V, Figure 12).

28. To further assess the effect of A7-SFV infection on functions of murine macrophages, spleen macrophages were prepared at different times after A7-SFV infection and used as antigen presenting cells to present OVA to antigen specific T cells. A7-SFV infection augmented the antigen presenting ability of spleen macrophages at day 1 after infection. Afterwards, no appreciable difference was found between cells isolated from mock- or A7-SFV-infected mice. This data indicate that A7-SFV infection can transiently activate antigen presenting cells *in vivo*, and trigger an autoimmunological response (Chapter V, Figure 13).

The following discussion will elaborate the above results and compare the data with those currently found in the literature.

Rationale of the Study

The rationale to study the effect of virus infection on the development of demyelination in an EAE model is the fact that the etiology of a common demyelinating disease, multiple sclerosis (MS) is unknown and its pathogenesis only partially revealed (2, 192 - 194). The genetic susceptibility of the individual plays a role in induction

of MS but, enviromental factors, especially infectious agents may be responsible for triggering the disease (192-194). Among numerous agents which have been suggested in the etiology of MS, viruses have been listed as important (83, 88, 168, 193, 195). In the 1960s, Adam and Imagawa took the first approach in investigating the possible link between virus infection and MS (196). In the 1970s, Norrby and Salmi were pioneers in further searching for and revealing that a relationship between virus infection and MS may exist, since elevated level of antibodies to certain viruses, i. e. measles, mumps and rubella viruses were found in the cerebrospinal fluid (CSF) and/or serum of MS patients (83, 88, 195, 197, 198). Recently, McFarland *et al.* found that impaired activity of measles virus specific, class II restricted cytotoxic T lymphocytes but not mumps virus specific CTLs were found in MS patients (84). However, all these clinical investigations suffered from a lack of evidence for a direct relationship between the history of viral infection and disease onset, and contradictory data have been reported in different studies (83, 88, 195, 197, 198). Therefore, these clinical investigation data need to be interpreted with great caution.

Sibley and his collaborators made an important contribution to the understanding of the pathogenesis of new exacerbations of MS patients (87). Their eight-year follow-up studies revealed that more than 70% of new

exacerbations are preceded by virus infections. Based on their observations, Sibley *et al.* suggest that exacerbation of MS are frequently caused by virus infections (87).

Because of limitations and constraints of clinical investigations, suitable animal models are necessary for understanding the pathogenesis of MS (1, 2, 192, 193). Experimental allergic encephalomyelitis, an autoimmune demyelinating disease in animals, is well accepted as a good model for pathogenesis studies (1, 2). Effect of virus infection on the disease onset and development have been studied (99-102). Both prevention and promotion of EAE have been reported. Lactic Dehydrogenase-elevating virus has been shown to up- or down regulate the disease (101, 102). However, little information is available about the possible mechanism of the effect of virus infection on EAE. The starting point of this project was, therefore, to establish a mouse model which could be manipulated to observe the effect of virus infection, and study the immunological basis of this effect.

Induction of EAE in BALB/c Mice

The susceptibility to EAE induction in mice can be divided into two categories, i. e., genetically resistant and genetically susceptible strains (14-18). The SJL/J mouse strain is a well known susceptible strain, under optimal conditions 100% of mice develop EAE after neuroantigen injection. Valuable information about

pathological and neurological features have been obtained from this mouse strain (2). The BALB/c mouse strain, in contrast, is a resistant strain and neurologic signs can not be observed after neuroantigen inoculation (12, 19). Lando *et al.* used low dose irradiation or cyclophosphamide treatment to render the BALB/c mice susceptible to EAE induction (19 - 22). Either treatment can change the resistant status of BALB/c mice, and about 50% of the mice develop neurologic signs under their experimental condition (19). Low dose irradiation was adapted in this project to treat BALB/c mice 2 days before neuroantigen inoculation and in a reproducible manner, only 20 - 30% mice developed typical neurologic signs about 24 days after neuroantigen inoculation.

The difference in disease onset between Lando's and this [illegible] may be the result of several factors: First, the susceptibility of different substrains of BALB/c mice may vary (67), and also the sex, strain and age are critical elements in EAE induction (14-18). Therefore, although the BALB/c mouse strain was used in these two studies, varied susceptibility was not unexpected. Second, the rating method of neurologic signs may also directly reflect the different rate of EAE incidence. Some mice demonstrated fur ruffing, tail atonia, but recovered afterwards, and these mice were not rated as disease positive in this work since the symptom was mild and lasted only for short period. Third, some technical variation between the different

laboratories and operators may also effect the results. For the above reasons, it was not unexpected that the quantitative differences in the incidence of EAE onset were found. A low percentage of incidence and much delayed disease onset were two unique features in this BALB/c mouse model. These features were advantageous when the effect of viral infection on these two parameters in this model were observed.

A7-SFV Infection Potentiated the EAE in BALB/c Mice

A non-lethal strain of Semliki forest virus, A7-SFV, was chosen to infect BALB/c mice. Strain A7 does not kill mice older than 19 days (127), but instead causes demyelination in a small portion of mice at about day 14 after infection (160). This virus strain has the advantage of not killing mice but also has been well characterized biologically, and biochemically (142-153, 157, 160-164). The immunopathogenesis of this virus is also a current topic in SFV studies (161-164). A7-SFV was used to infect BALB/c mice at different days before and after neuroantigen inoculation. To avoid direct disturbance of the blood-brain barrier by virus inoculation (160), intraperitoneal injection was chosen as the infection route.

A7-SFV infection of mice at day 7 after neuroantigen inoculation had a maximal effect on the EAE induction and development. Not only was the time of disease onset drastically shortened by virus infection from 24 days to 14

days, but the incidence of EAE onset was also increased from 20-30% to 60-80%. Both virus infection and low dose irradiation were necessary in rendering BALB/c mice more susceptible to the EAE induction, as no disease could be induced in mice treated with neuroantigen and virus but without 350 R irradiation (Table 3). Low dose irradiation may inactivate the radiosensitive T suppressor cells (19), therefore weakening the resistant status of mouse. Only one mouse was killed by virus infection out of 55 mice in the non-irradiated group. As expected, virus infection alone after 350 R irradiation increased the disease onset and the death (from 1.8 to 12%) at about 8 - 9 days after infection (Table 3). This was about 5 days later than that of mice treated with both neuroantigen and virus infection. Therefore, virus-induced disease in a small portion of mice could be easily distinguished from EAE induced by neuroantigen inoculation and potentiated by virus infection.

The potentiation effect was critically time dependent. When virus was inoculated at day 3 after neuroantigen inoculation, no potentiation effect was observed (Table 4). The timing effect may be directly related to the replication status of the virus and immunological status of the host. Timing has also been found to be important in other virus models (100-102). Lactic Dehydrogenase-elevating virus infection at different times can have both an up- and down regulating effect on EAE. This effect may

be related to killing of antigen presenting cells (Ia+) by the virus. A diminished number of Ia+ cells may prevent disease at certain times after infection as the antigen presenting process is less efficient (102'. Under certain circumstance, killing of macrophages may also release cellular factors such as TNFα, proteases, oxygen radicals, etc., which may promote demyelination locally, since these substances may be partially responsible for the demyelination (1, 2, 199). Killing of macrophages may also reduce the number of cells producing IFNα,β, which may also be responsible for the decrease of disease incidence as it was shown recently that *in vivo* administration of IFNα,β can down-regulate the rate of EAE onset (215). These interpretations may not necessarily be applicable to this EAE model, but, the data suggest that the basis of this potentiation effect may be immunologically mediated.

Dose dependence was another feature of this potentiation effect. An inoculum of 10^6 PFU per mouse produced the highest potentiation effect but 10^8 PFU per mouse did not increase the EAE incidence. One explanation for this could be interference of defective-interfering (DI) particles with the replication of virus, therefore affecting the potentiation effect. In this respect, Semliki forest virus is well known for the production of DI particle when a high inoculum is used, and this may be partially responsible for the switching from lytic to persistent infection (153).

An important question to address was whether virus replication was necessary for the potentiation effect. When the same amount of U. V. inactivated virus was used to replace infectious virus, no potentiation could be observed. Therefore it was concluded that virus replication was mandatory for the potentiation effect on EAE induction in the BALB/c mouse strain.

Preceding A7-SFV Infection Did Not Potentiate the EAE

During the development of this model, a report by Mokhtarian and Swoveland told a different story on another genetically resistant mouse strain C57B1/6 (115). When they predisposed C57B1/6 mice to A7-SFV infection, EAE was induced with myelin basic protein or MBP-primed lymph node cells from a syngeneic donor 2 to 4 weeks after virus infection. This effect could only be mediated by virus infection since U V inactivated virus had no effect. In this work, it was observed that in both BALB/c and SJL/J mouse strains that predisposing mice to A7 infection 10 days before neuroantigen inoculation actually decreased the incidence of EAE onset (Table 8). This was obviously contradictory to the mentioned report (115). To address the discrepancy, the same protocol used by Mokhtarian and Swoveland was used in the BALB/c mouse model (Table 7). BALB/c mice were infected with A7-SFV virus 21 days before neuroantigen inoculation with or without 350 R treatment. In either case, no EAE was induced in the BALB/c mouse

strain. This discrepancy could be explained by several facts. First, even though BALB/c and C57B1/6 both are genetically resistant strains, they may differ very much in terms of susceptibility to EAE induction, and/or the nature of this resistance (14-18). Second, different substrains may also deviate from the original A774 virus strain, therefore having a different pathogenetic outcome in mice, especially when viruses used in these two laboratories were grown in different tissues (115, 200). Third, different neuroantigens used may also affect the induction of disease. In Mokhtarian's report, MBP was used, while in this project, total mouse spinal cord homogenate was used. In particular, different mouse strains may vary in their susceptibility to different encephalitogens, e. g., it has been observed that only some substrains of BALB/c mice are sensitive to DM-20, a fraction of proteolipid apoprotein (PLP) (67). Different effects on EAE induction with the same virus have also been observed with Lactic Dehydrogenase-elevating virus (101, 102). It should be emphasized, therefore that viral pathogenesis may be varied under different experimental conditions.

Mechanisms of EAE Potentiation by A7-SFV in BALB/c Mice

Several virologically-mediated mechanisms could account for this potentiation effect caused by viral infection: 1. Molecular mimicry may play a role in the virus-induced potentiation effect (167). The observation

that autoantibody against myelin glycolipid crossreacts with SFV is revelant to this work (105, 106). Therefore, virus infection may trigger production of autoreactive T cells or B cells, and autoantibodies or autoreactive CTL may harm innocent by-stander neurons or oligodendrocytes. 2. Virus infection may kill cells in the CNS, e. g., Theiler's murine encephalomyelitis virus may cause demyelination by killing oligodendrocytes, a type of cell which is responsible for remyelination (92-94); 3. A persistent infection in the CNS may potentiate autoimmune demyelination by providing a continuous stimulation of autoimmune cells. A recent report showing that SFV antigen may be found in astrocytes for a relatively long time supports this possibility (202);

Some evidence seems to support the involvement of molecular mimicry in this model. First, Webb *et al.* showed that autoantibodies against myelin glycolipid crossreact with SFV (105, 106). Second, lymphocytes from A7-SFV infected mice had responses to BP in this work (Table 10); Third, Mokhtarian *et al* suggested that molecular mimicry may be the mechanism in their model (115). However, several facts are against the mechanism of molecular mimicry in this model. First, when BALB/c mice were predisposed to A7-SFV infection 21 days before neuroantigen inoculation as in Mokhtarian's work, no EAE was induced. Second, BP may not be a major encephalitogen in BALB/c mouse strain (67). Third, autoreactive antibodies in SFV infected mice were

usually found at 7 days or later p. i. (106). In this model, the potentiation effect was observed 5 days after A7-SFV infection. Fourth, if the potentiation is mediated by molecular mimicry, one may suspect that 10^2 and 10^8 inocula should have the similar potentiation effect as 10^6 inoculum did. However that is not the case in this model. Therefore, even the possibility of molecular mimicry can not be excluded in this model, it may not be the only mechanism operating in the EAE potentiation induced by A7-SFV infection.

Some mutants of SFV, e. g., M9, kill oligodendrocytes *in vivo* (159), but the A7-SFV strain does not (130). There is no evidence concerning the killing of neurons by the A7 strain either (130). A7-SFV replication in both blood and brain is a very short event (157, 160). In fact, no maturation of viral particles has been observed by electron microscopic examinations (157, 160, 201), even though viral antigen was found in astrocytes for long periods after disappearance of infectious virus (202).

A7-SFV replication in the CNS of BALB/c mice was shown to be a short event in this model. Infectious virus was no longer detectable at day 8 after inoculation. Low dose irradiation two days before virus infection prolonged the time of production of infectious virus from 7 days to 10 days, and production of infectious virus at day 5 was one log higher than with the non-irradiated control. Viremia was prolonged and enhanced by 350 R irradiation (Table 15).

However, when virus infection was carried out at day 9 after neuroantigen inoculation, no significant difference was found. Therefore, at the time of disease onset, virus replication in the CNS actually had declined to a very low level. Even though infectious virus is not detected, viral antigen or viral RNA could still exist (202). To answer this question, total brain RNA was extracted and hybridized with cDNA probes corresponding to the E1 or E2-E3 region, the virus spike protein (203) necessary for maturation of infectious virus (203). No viral RNA could be detected at day 12 after viral infection. As the hybridization methods have a limitation in sensitivity, a low level of SFV may still persist in the CNS of BALB/c mice. Recent data by Mokhtarian *et al* (115) who used *in situ* hybridization techniques to detect SFV RNA, which no persistence of viral RNA was found, is in agreement with this work.

From investigation of viral infection *in vivo*, it was concluded that A7-SFV may not persist in the CNS and lymphoid cells *in vivo*, however, low level production of infectious virus was still detected in CNS at the time of disease onset. It was impossible to exclude that a part of this potentiation effect was mediated by virus killing of brain cells. Therefore, another approaches were taken to search for the mechanism of this potentiation effect.

Transfer of EAE

It is well known that demyelination in EAE can be

transferred to naive mice by neuroantigen primed lymph node cells or spleen cells (1, 2). L3T4+ cells were shown to be the principal element in this disease transfer (38, 42). The presumption was made that if the potentiation effect was mediated by immune cells, the disease should also be transferred to naive mice by immune cells. When spleen or lymph node cells were prepared from mice 10 or 12 days after neuroantigen injection, 3 or 5 days after virus infection, and incubated in the presence of an optimal concentration of ConA, and live cells transferred to naive mice, about 20% of mice developed typical paralysis 12 days after transfer (Table 21). Cells from mice treated with neuroantigen or virus only did not induce disease. Transmission of virus by these cells to naive mice was excluded since no infectious virus was detected by infectious center assay.

The incidence of disease onset in recipients was low (20%) and about the same percentage was obtained in four separate experiments. Therefore, the low percentage of EAE induction could not be for technical reasons, but may be related to the non-responder status of the BALB/c mouse strain. It is also possible that the effect of A7-SFV infection on EAE onset can not be completely transferred by these spleen or lymph node cells from MSCH primed and A7-SFV infected mice. Another point to be made is that in some mouse EAE models, $1x10^7$ ConA activated cells can transfer the disease (35, 115). In this model, a minimum number of

$2x10^7$ cells per mouse was needed. This again may be related to the genetic resistance of the BALB/c mouse strain. Another possibility is that antigen stimulated cells may be more efficient than mitogen activated cells. Myelin basic protein was also used to replace ConA in *in vitro* stimulation. The disease was also induced in naive mice by these cells, but the incidence rate of disease onset remained unchanged. Two explanations should be considered. First, the low EAE incidence may still be related to the non-responder status of BALB/c mice; second, MBP may not be the only encephalitogen in this system since lymph node cells from mice treated with both neuroantigen and virus did not proliferate very well when stimulated with BP (Table 10, 67, 70). The majority of transferred cells expressed L3T4 phenotype (70%). More than 99% of cells were Thy. 1 positive. This data agreed with a general notion that induction of EAE in naive mice was mediated by T cells, especially L3T4 T cells (38, 42).

Effector Cells in EAE in This Model

To directly investigate the role of L3T4+, Lyt.2+ and macrophages in disease induction in this model, *in vivo* administration of different MAbs was used to observe the modulation effect. The result was not straightforward, since about 20-30% of mice died after administration of MAbs and virus infection. Two interpretations can be given to reveal the nature of this phenomenon. First, death may

be caused by an acute virus infection, after elimination of effector cells capable of limiting virus infection. This is supported by the fact that the same death rate was found in infected mice treated with MAbs but without neuroantigen. No death was found in mice treated with MAbs only. Second, death was observed in mice treated either with anti-L3T4 or anti-Lyt.2 MAb, but only anti-L3T4 MAb prevented mice from developing neurologic signs of EAE. Administration of F4/80 also prevented mice from developing neurologic signs of EAE. Since F4/80 targets resident and responsive macrophages in mice, it was not unexpected to see this effect, as the role of macrophages in EAE induction and development is well known (1, 48). Therefore, it was confirmed that L3T4 T cells and macrophages but not Lyt. 2+ T cells played a central role in EAE induction also in this model.

Cytokine Production in BALB/c Mice after A7-SFV Infection

How is the potentiation effect mediated immunologically through a virus infection? Several possibilities should be considered. First, virus infection may kill specifically T suppressor cells and thereby, changing the tolerant stage towards autoantigens in genetically resistant mouse strains (205). As demonstrated in the vesicular stomatitis virus system, a tolerant status to TNP in mice could be broken by VSV infection, as VSV killed T suppressor inducer cells (205). Second, virus infection activates immune cells, i.

e., macrophages, T cells and B cells. These activated cells release regulator molecules, such as IFNα,β, IFNγ, IL-1, IL-2, etc., and further activate or potentiate the functions of autoimmune cells. Third, virus infection may activate mainly antigen presenting cells, especially macrophages. These activated antigen presenting cells may have preference in presenting autoantigens to autoimmune cells, thereby upregulating the disease (126). Fourth, both autoantigen specific T cells and antigen presenting cells may be activated by virus infection via an unknown mechanism.

In vitro investigation of infection of different subpopulations of mouse spleen cells demonstrated that Mac-1+ cells in the total spleen or adherent cell population were preferentialy infected by SFV. Macrophages, not T or B cells, were the only target of SFV among the immune cells. Even in macrophages, this infection was abortive, because no infectious virus was released from infected cells. Even though both Ia+ and Ia- macrophages were infected by SFV (Figure 6), neither killing of cells nor CPE was found (Tables 18, 19 and 20). Therefore this potentiation effect may not be caused by deletion of immune cells such as T cells and macrophages.

To investigate the second possibility, two experimental apporaches were used. The first approach was to investigate the production of certain cytokines after virus infection *in vivo*. IFNγ, α, β, IL-1, IL-2 are all well known and

characterized cytokines in terms of function, biochemical, biological and molecular, genetical nature (172, 173, 206-211). IFNα,β is induced by virus infection as demonstrated in many virus models (135, 162), and certainly plays an important role in limiting virus replication and transmission (212-214). IFNγ can best be regarded as a T cell growth promoting factor and regulator (208), up- and down regulating of immune responses depending on the conditions (206, 215). IFNγ is known for its effect on induction of class II antigen expression on multiple cell types, i.e., T lymphocytes, macrophages, astrocytes, endothelial cells, etc (119, 126). Since a class II MHC molecule is needed in T cell recognition and activation as a component of the MHC-Ag-TCR (T cell antigen receptor) complex, it is assumed that elevated class II antigen molecule expression *in vivo* by enhanced production of IFNγ could be a precipitating factor in certain autoimmune diseases (126).

IFN Production

Investigation of IFN production after SFV infection *in vivo* was carried out in BALB/c mice. High level production of IFNs was found at day 1 to day 4 after infection, especially in lymph node cells and peritoneal cells. This is expected since SFV is known to be a good inducer of IFNs (134 - 138, 162 - 164). IFNα,β is produced in an earlier phase of infection, and IFNγ is produced later and released

by T lymphocytes, such as CTL cells (135, 162). Therefore, virus infection may activate immune cells and trigger the production of IFNs, which may up- or down regulate the autoimmune disease.

In vivo Administration of IFNγ in Neuroantigen Primed Mice

Since measurement of IFNs production *in vitro* after viral infection may not reflect the *in vivo* situation and is only an indirect approach for studying the effect of IFNs produced *in vivo*, it may not be relevant from the pathogenesis point of view. To directly test the effect of IFN *in vivo*, recombinant IFNγ was administered to BALB/c mice which had received neuroantigen 10 days previously. The time of administration of IFNγ was based on the observation that most abundant IFNs were produced at day 3 or day 4 after virus infection *in vivo*. A dose of 10,000 U was injected into each mouse at each time to ensure a high enough dosage. One may expect that IFNγ potentiates the disease (125) as did the infectious virus. However, this was not the case. *In vivo* administration of IFNγ did not potentiate the disease. Two explanations may be considered; First, systemic administration of IFNγ may have a different effect from locally produced IFNγ (215). Locally presented IFNγ may favor the activation of antigen presenting cells, i. e., macrophages, dendritic cells, or other type of cells which have antigen presenting ability, such as astrocytes and endothelial cells and therefore, increase the number

of antigen presenting cells and the density of MHC molecules on these antigen presenting cells. However, systemic administration may activate different cell populations. Some cell populations may have a feedback effect, even down-regulating the function of L3T4 cells. In particular, it is known that only a small number of effector cells are needed to initiate EAE (204). The actual mechanism may not be so simple, and can not be easily elucidated.

Downregulation of EAE by *in vivo* administration of IFNγ reported in a rat EAE model (217) was also confirmed recently in another mouse EAE model (215). In both high responder and low responder strains, *in vivo* administration of recombinant IFNγ decreased the rate of EAE onset and anti-IFNγ MAb exacerbated the disease, which is similar as observed in this model. The second explanation may be based on recent reports on transgenic mice (218). A gene which encodes $I\text{-}A^d$ or $I\text{-}E^d$, or class I antigen were introduced to transgenic mice which were used to study the effect of increased MHC molecules on induction and development of insulin dependent diabetes, which is an autoimmune disease. In these models, histopathological examinations demonstrated that no lesion was found on β-islet cells, the target of autoimmune cells expressing high levels of Ia antigens in these mice (218). Therefore, an enhanced level of class II antigen may not be responsible for autoimmune destruction of β cells. The diabetes induced in these

transgenic mice may be caused by intracellular association of the MHC antigen molecule and insulin, which leads to decreased net production of insulin (218). Therefore, the results suggest that the expression of the MHC molecule may not necessarily correlate with the antigen presenting ability. The results of *in vivo* administration of IFNγ in this model indicate that increased production of IFNs after viral infection is not directly responsible or can not be the only factor for this virus induced potentiation effect on EAE.

IL-2 Production

Production of IL-2 by different cell populations after viral infection was also enhanced (Table 24). The IL-2 level varied among different cell populations and at different times, but with a general trend of increased production. Higher production of IL-2 may be explained by a primary *in vivo* stimulation of virus infection. The result also agreed with the general notion that production of IL-2 and IFNγ are tightly connected (219, 220). However, the significance of elevated IL-2 production is not known, and can only be studied by *in vivo* administration of IL-2. This issue was not addressed in this project since purified IL-2 was not available for this work.

Based on *in vitro* data, one may suggest that elevated IL-2 could have a dual effect in EAE development. IL-2 is necessary for T cell development from G1 to S phase (221).

Afterwards it may promote the proliferation of L3T4 T cells sensitized by neuroantigen injection, and amplify the autoimmune T cells and exacerbate the disease. However, it is also known that IL-2 can drive oligodendrocytes to proliferate, thereby promoting the remyelination process, an essential event in recovering from acute EAE (124). IL-2 could down regulate the disease as well. Both possibilities can be approached experimentally but were not done during these studies.

IL-1 Production

IL-1 production *in vivo* in BALB/c mice after A7-SFV infection was variable. Decreased IL-1 production in peritoneal cells was found at day 3 or 4 after virus infection *in vivo*. This contradicted the evidence that production of IL-2 or IFNs were increased at the same time. As IL-1 is needed for T cells to proliferate (210), and is an early event in cell activation, the release of IL-1 by macrophages may be a transient and initial event. Further experiment was done to further investigate the kinetics of IL-1 production after A7-SFV infection. Since virus was inoculated i. p., peritoneal cells were the first to encounter virus infection, and the impact of virus infection on these cells may also affect later events in other immune cell populations. To address this question, production of IL-1 by peritoneal cells was investigated from day 1 after A7-SFV infection. At day 1 post-

infection, the most abundant production of IL-1 (200% of mock-infected control) was found. The high level of IL-1 lasted until day 2 p. i. and declined afterwards.

Several explanations to the observed IL-1 production in peritoneal cells can be considered. First, elevated production of IL-1 at day 1 or 2 after virus infection may result from *in vivo* stimulation by viral infection. Similarly AIDS patients have a spontaneous high level production of IL-1 which can be contributed to *in vivo* exposure to HIV (222). It has also been reported that peritoneal cytotoxic macrophages can be found at 24 h after SFV infection *in vivo*, then disappear quickly, after which virus specific CTL can be isolated (136). The nature of this SFV activated cytotoxic macrophages is unknown, but enhanced IL-1 production may be the result of by these activated macrophages. Second, the decreased level of IL-1 after day 2 of virus infection could be caused by degeneration of IL-1 which was not needed at this stage. Alteratively, prostaglandin E2, a known inhibitor of the cyclooxygenase pathway, may play a role in inhibiting IL-1 production (223). Third, decreased IL-1 level could also be caused by an IL-1 inhibitor released after virus infection. Down regulation of IL-1 by viral infection has been reported in some virus infections (224, 225). Inhibition may be caused by IL-1 inhibitor released by macrophages after virus infection (224). SFV infection of spleen mononuclear cells also triggers an IL-1 inhibitor *in vitro*

(226). The inhibitor is produced at a later stage of spleen mononuclear cell proliferation, and it affects the mitogen driven proliferation of spleen cells (226). This inhibitor may also be reponsible for the decreased level of IL-1 after day 2 of virus infection.

Transiently enhanced production of IL-1 can activate the immune regulatory network. Furthermore, IL-1 may also directly affect functions of CNS cells in the demyelination process (209), since IL-1 can be released by some cell types in the CNS, eg., microglial cells and astrocytes (118, 120), and IL-1 can also modulate the differentiation and function of these cells (123). However, to address the question whether IL-1 can directly affect the onset or development of the demyelination process can be approached by *in vivo* administration (systemic or local) of IL-1.

From *in vitro* investigations of cytokine production, it is safe to say that viral infection caused a general change of cytokine production and regulation. SFV infection can activate immune cells in a time dependent manner. These activated cells or released immune regulatory molecules may participate in potentiating demyelination. Nevertheless, other mechanism(s) may also operate here, since *in vivo* administration of recombinant IFNγ did not potentiate the disease. Cells of the lymphatic system circulate and migrate through BBB. These circulating cells may also take residence in the CNS, and if activated, could initiate or potentiate CNS demyelinating disease by releasing

inflammatory moleculess and cytokines. Recent studies demonstrate that TNFα released by activated macrophages can directly cause demyelination in neurons *in vivo* and *in vitro* (199).

Potentiation of EAE by Spleen Cells Prepared from A7-SFV Infected Mice

Since recombinant IFNγ cannot replace A7-SFV infection at day 7 for the potentiation effect, the effect of virus infection may not be mediated solely by soluble factors. Another approach was taken to determine whether *in vivo* A7-SFV primed spleen cells can replace virus infection. Spleen cells were taken from mice infected by A7-SFV five days after infection, and inoculated i. v. into mice which had received neuroantigen 7 days before cell transfer. Mice which received cells from A7-SFV infected donors developed EAE at about day 14 to 17 after neuroantigen inoculation, i. e., 7 to 10 days after cell transfer. The incidence of disease onset was between 45 - 60% in four experiments. In contrast, inoculation of the same number of normal spleen cells had no effect. It was evident that A7-SFV primed cells can also shorten the time of disease onset and slightly increase the rate of disease onset. Transferred cells were also examined for the existence of infectious virus, but none was detected with an infectious center assay. When the same number of cells from A7-SFV primed mice were transferred into normal mice at the same time no

disease developed. Therefore it could be concluded that disease potentiation by A7-SFV primed cells was not caused either by residual infectious virus or the transferred cells only, but must be induced by two populations of cells, one is the host (designated as A), another from the donor (designated as B). An interaction between cells was necessary since supernatants from ConA stimulated A7 primed spleen cells did not have an effect. It was assumed that population A must consist of neuroantigen primed autoreactive L3T4 T cells, since they are the effector cells in EAE induction.

Spleen cells from A7-SFV primed mice were depleted of B cells and transferred to neuroantigen primed mice but this procedure did not abolish the potentiation effect. Since depletion of B cells by a T cell column did not have any effect, B lymphocytes seem not play a role in this potentiation effect. When cells were adhered to plastic plates for one hour, the potentiation effect was abolished. Depletion by adherence can not completely remove macrophages as also shown with the fluorescence. However, this process did significantly decrease the number of macrophages in spleen cells, indicating that macrophages play an essential role in the potentiation effect. When spleen cells were irradiated with 1500 R and transferred, only one out of nine mice developed the disease.

It was likely from the depletion experiments that both radiosensitive T cells and macrophages were required for

the potentiation effect. Therefore, it would be important to analyse the coordination and interaction between T cells and macrophages in this model. Although the cellular basis is known, the question is very difficult to answer. Several possibilities should be considered: First, virus infection may activate macrophages, and certain subsets of macrophages may preferentially act as autoantigen presenting cells, and activate autoantigen specific T cells (244); second, activated macrophages could release greater amounts of plasminogen activator, which may influence the permeability of BBB (242) and facilitate the penetration of autoreactive T lymphocytes; third, activated macrophages taking residence in the CNS, may activate local antigen presenting cells, and attract circulating autoreactive T cells, and promoting initiation of local immunological responses (118 - 125). Alternatively, activated macrophages may release certain substances, i. e., TNFα, IL-1 etc, which may have a direct effect on demyelination (2). All these interactions can be carried out by activated macrophages and T cells; fourth, virus infection may induce virus specific CTL, which are crossreactive to CNS myelin, thereby initiating the demyelination (167, 245); fifth, virus infection may specifically induce a subset of T cells, which may be lacking or suppressed in neuroantigen primed mice, since it is known that macrophages can induce either T helper or T suppressor cells *in vivo* (240). Transfer of the T cells may synergistically act with

another subset of T cells in neuroantigen primed mice, and both subsets of T cells can induce demyelination. It is also likely that activation of transferred T cells requires help of macrophages, especially as it is known that inflammatory/Th1 T cells require a high ligand density which may be provided by macrophages infected by intracellular pathogens (244). Nevertheless, all of these possibilities need to be tested experimentally.

In vitro and in vivo Studies of the Effect of A7-SFV Infection on Functions of Murine Macrophages

Further experiments were carried out *in vitro* to investigate how the capacity and function of macrophages is influenced by A7 infection, which may have relevance to this potentiation effect *in vivo*. I used two macrophage populations which have been well characterized and easily obtained, namely, thioglycollate elicited peritoneal macrophages, and bone marrow-derived macrophages. In both macrophage populations, a limited replication of virus was found, as even at the peak level of replication, only one infectious virus was produced per 10 infected macrophages. Since residual membrane associated viral particles were neutralized by polyclonal anti-SFV antiserum at 4°C after virus adsorption, the detected infectious virus was newly produced. Within the whole observation period, no cytopathic effect was found. In contrast to some other viruses, e. g., measles virus (227, 228), SFV replication

did not change when macrophages were activated. However, virus replication was associated with the differentiation stage, because the least mature macrophages in BMDM were resistant to virus infection.

Two criteria were used to evaluate the effect of virus infection on the functions of macrophages, i. e., interleukin-1 production and class II antigen expression. A transient and rapid induction of IL-1 in resting thioglycollate elicited peritoneal macrophages was observed with both wild type and A7 strains of SFV. The induction effect was also dose dependent since a more pronounced induction effect was found in macrophages infected with higher m. o. i. Since macrophages are evidently semi-permissive for SFV replication, it is not unexpected that a greater amount of input virus is needed for a larger induction effect. As UV inactivated virus did not induce IL-1 production, virus replication was required for the inducing effect. In mock infected macrophages, a small amount of IL-1 was also produced at 24 h after mock infection. Here, the stimulus may be derived from newly replaced medium, especially 2-mercaptoethanol, a component of the medium shown to have a synergistic effect in IL-1 production and T, B cell activation (229).

Both SFV strains induced Ia antigen expression on PM in a time and dose dependent manner. A small percentage of thioglycollate elicited peritoneal macrophages are known to express Ia antigens (230). The level of Ia antigen on PM

decreased gradually after seeding to plastic plates, and at 16 h after seeding, Ia antigen became almost undetectable (230). When mock-infected PM were incubated for 48 h *in vitro*, after which only 2-5% PMs were Ia antigen positive. However Ia antigen expression on virus infected PM reached a maximal level at 24 h after infection, and declined afterwards. The kinetics of virus induced Ia antigen expression is different from rat ConA supernatant or IFNγ, which increased timewise over a much longer time.

Several possible mechanisms of virus induced Ia antigen expression on PM should be considered. First, the induction effect may be caused by a receptor-ligand like interaction, which subsequently activates Ia antigen synthesis machinery. If the presumption is right, UV inactivated virus, or in the form of purified viral proteins, should have the same effect as infectious virus. Second, it is known that membrane molecules in macrophages continuously recycle (231), and regeneration of some membrane molecules takes about 8 h *in vitro* (232). Virus infection and replication may disturb this network, and up or down regulate the synthesis and/or recycling of certain membrane molecules under some circumstances (233). In this case, virus replication may be required to have effect on Ia antigen expression. Third, both possibilities could exist simultaneously. These possibilities were not studied in depth in this work.

It was found that virus infection upregulated the

antigen presenting ability of spleen macrophages. The most pronounced effect was observed at day 1 after A7 infection *in vivo*. A 50% increase of response to antigen was found at day 1 after virus infection *in vivo* with irradiated spleen cells as antigen presenting cells. Upregulated antigen presenting ability may derive from an increased number of Ia+ macrophages or increased density of Ia antigens on antigen presenting cells. Since macrophages are a very heterogeneous population (234), it is quite possible that this increased antigen presenting ability is associated only with a subset of macrophages infected by virus. These macrophages may also behave as cytotoxic macrophages and inflammatory cells in this autoimmune disease process, since they also release high levels of IL-1, after viral infection *in vivo*. It is quite possible that these activated macrophages selectively activate certain subset of CD4+ T cells, i. e., inflammatory CD4+ T cells, to initiate inflammatory reaction in the CNS, subsequently leading to demyelination.

It would be oversimplified to relate the *in vitro* data to the *in vivo* situation, especially since the extent of binding, internalization and processing of antigen by an APC may differ *in vitro* and *in vivo* as a result of the local concentration and form of the antigen encounted by an APC (241). However the data may give clues to understanding the pathogenesis. In this case, it is feasible to suspect that an important role of virus infected macrophages in

potentiating EAE in genetically resistant BALB/c mice may be related to an upregulated antigen presenting ability at or during a certain time period.

Potentiation Effect is Independent of SFV Neurotropism

Potentiation of EAE by SFV infection may be restricted to this autoimmune disease only and be related to the neurotropism of this virus. Three pieces of evidence suggest that it may not be the case. First, SFV infection can sustain immunity to OVA in BALB/c mice for a much longer time than in uninfected mice (Fig. 12). Second, when SFV was introduced into another autoimmune disease model - experimental arthritis, SFV infection also sustained the DTH reaction to autoantigen - collagen for a much longer time than in mock-infected mice (Dr. B. Rubin, personal communication). Third, and maybe the most convincing evidence, was derived from the observation of A7-SFV infection in an OVA-oral tolerance model in BALB/c mice. When A7-SFV was introduced into mice which were rendered tolerant to OVA, the tolerant status was abolished by virus infection. A strong DTH reaction was induced at 24 h after virus infection and lasted for a longer time than non-tolerant OVA immunized mice (Dr. D. R. Green, personal communication). It seemed that SFV infection could exert an effect on immune cells, and upregulate or sustain an autoimmune reaction. The mechanism in both these cases has not yet been approached, but the data indeed support the

concept that SFV infection may alter the immuneregulatory network and cause an aberrant immune reaction and subsequently potentiating an autoimmune disease.

Conclusions

In conclusion, this thesis project therefore not only established a suitable mouse model for studing the new excerbation of human demyelinating disease - MS, but also characterized the basis of this model. It was demonstrated that virus infection can potentiate an autoimmune disease by an immunologically mediated mechanism. The EAE potentiated by virus infection was transferred to naive animals. The potentiation effect was caused by virus primed immune cells, specifically, radiosensitive T cells and macrophages. This work also demonstrated that virus infection can alter the capacity and function of macrophages, and upregulate antigen presenting ability on a certain subset of macrophages, which could be a precipitating factor in potentiating an autoimmune disease. Therefore, data presented in this project adds a new aspect on the discussions of the mechanism of virus potentiated autoimmune disease, and opens an important new avenue for studies of the immunopathogenetic of the effect of virus infection on autoimmunity. The concept of immune regulation disturbance by virus infection is applicable to other autoimmune disease models as well. More detailed studies on aspects of the immunological events during virus-induced

immune modulation are necessary for a better understanding of the phenomena described in this work.

REFERENCES

1. Paterson, P. Y. 1976. Experimental autoimmune (allergic) encephalomyelitis. In: P.A.Miescher and J.K. Mueler-Eberhard (Eds.). Textbook of Immunopathology. Grune and Scratton. New York. p. 179.
2. Raine, C. S. 1984. Biology of disease-analysis of autoimmune demyelination: Its impact upon multiple sclerosis. Lab. Invest. 50: 608.
3. Lewis, J. H. 1934. Antigenic relationship of the alcohol-soluble fractions of brain and testicle. J. Immunol. 27: 473.
4. Rivers, T. M., and F. F. Schwentker. 1935. Encephalomyelitis accompained by myelin destruction experimentally produced in monkeys. J. Exp. Med. 61: 689.
5. Rivers, T. M., D. H. Sprunt, and G. P. Berry. 1933. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. J. Exp. Med. 58: 39.
6. Freund, J., E. R. Stern, and T. M. Pisani. 1947. Isollergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion. J. Immunol. 57: 179.
7. Morgan, I., 1947. Allergic encephalomyelitis in monkeys in response to injection of normal monkey nervous tissue. J. Exp. Med. 85: 131.
8. Kabat, E.A., A. Wolf, and A. E. Bezer. 1947. Rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants. J. Exp. Med. 85: 117.
9. Lee, J. M., and P. K. Olitsky. 1955. Simple method for enhancing development of acute disseminated encephalomyelitis in mice. Proc. Soc. Exp. Biol. Med.

89: 263.

10. Wiener, S. L., M. Tinker, and W. L. Bradford. 1959. Experimental meingoencephalomyelitis produced by hemophilus pertussis. Arch. Pathol. 67: 694.
11. Maillard, J., and B. R. Bloom. 1972. Immunological adjuvants and mechanism of cell cooperation. J. Exp. Med. 136: 185.
12. Munoz, J. J., and I. R. Mackay. 1984. Production of experimental allergic encephalomyelitis with the aid of pertussigen in mouse strains considered genetically resistant. J. Neuroimmunol. 7: 91.
13. Linthicum, D.S., J. J. Munoz, and A. Blaskett. 1982. Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. Cell. Immunol. 73: 299.
14. Stone, S. H., E. M. Lerner, and J. H. Goode. 1969. Acute and chronic autoimmune encephalomyelitis: age,strain,and sex dependency. The importance of the source of antigen. Proc. Sco. Exp. Biol. Med. 132: 341.
15. Gasser, D. L., C. M. Newlin, J. Palm, and N. K. Gonatas. 1972. Genetic control of susceptibility to experimental allergic encephalomyelitis in rats. Science 181: 872.
16. Bernard, C. C. A. 1976. Experimental autoimmune encephalomyelitis in mice: Genetic control of susceptibility. J. Immunogen. 3: 263.
17. Montgomery, I. N., and H. C. Rauch. 1982. Experimental allergic encephalomyelitis (EAE) in mice: primary control of EAE susceptibility is outside the H-2 complex. J. Immunol. 128: 481.
18. Webb, C., D. Teitelbaum, R. Arnon, and M. Sela. 1973. Correlation between strain differences in susceptibility to experimental allergic

encephalomyelitis and the immune response to encephalitogenic protein in inbred guinea pigs. Immunol.Commun. 2: 185.

19. Lando, Z., D. Teitelbaum, and R. Arnon. 1980. Induction of experimental allergic encephalomyelitis in genetically resistant strains of mice. Nature 287: 551.
20. Lando, Z., D. Teitelbaum, and R. Arnon. 1979. Effect of cyclophosphamide on suppressor cell activity in mice unresponsive to EAE. J. Immunol. 129: 2156.
21. Lando, Z., D. Teitelbaum, and R. Arnon. 1979. Genetic control of susceptibility to experimental allergic encephalomyelitis in mice. Immunogenetics 9: 435.
22. Lando, Z., D. Teitelbaum, and R. Arnon, 1981. The immunologic response in mice unresponsive to experimental allergic encephalomyelitis. J. Immunol. 126. 1526.
23. Gonatas, N. K., and J. C. Howard. 1974. Inhibition of experimental allergic encephalomyelitis in rats severely depleted of T cells. Science 186: 839.
24. Sriram, S., and C. A. Roberts. 1986. Treatment of established chronic relapsing experimental allergic encephalomyelitis with anti-L3T4 antibodies. J. Immunol. 136: 4464.
25. Sedgnick, J. D., and D. W. Mason, 1986. The mechanism of inhibition of experimental allergic encephalomyelitis in the rat by monoclonal antibody against CD4. J. Neuroimmunol. 13: 217.
26. Levine, S., E. J. Wenk, and E. M. Hoenig. 1968. Passive transfer of allergic encephalomyelitis between inbred rat strains: correlation with transplantation antigens. Transplantation 5: 534.
27. Paterson. P. Y. 1960. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. J. Exp. Med. 111: 119.
28. Bernard, C. C. A., J. Leydon, and I. R. Mackey, 1976. T

cell necessity in the pathogenesis of experimental autoimmune encephalomyelitis in mice. Eur. J. Immunol. 6: 655.

29. Paterson, P. Y. 1963. Cells, antibodies and auto-immune disease. In Amos, B. and H. Koprowski, (Eds): Cell-bound antibodies. Philadelphia. The Wistar Institute Press. p. 101.
30. Paterson, P. Y., and N. C. Didakow. 1961. Transfer of allergic encephalomyelitis using splenectomized albino rats. Proc. Soc. Exp. Biol. Med. 108: 768.
31. Falk, G. A., M. W. Kies, and E. C. Alvord. 1969. Passive transfer of experimental allergic encephalomyelitis mechanisms of suppression. J.Immunol.103: 1248.
32. Stone, S. H. 1961. Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea pigs. Science 134: 619.
33. Stone, S. H. 1961. Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea pigs. Science 134: 619.
34. Astrom, K., and B. H. Waksman. 1962. The passive transfer of experimental allergic encephalomyelitis and neuritis with living lymphoid cells. J. Pathol. Bact. 83: 89.
35. Pettinelli, C. B., and D. E. McFarlin. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: Requirement for $Lyt1^{+}2^{-}$ T lymphocytes. J. Immunol. 127: 1420.
36. Holda, J. H., and R. H. Swanborg. 1982. Autoimmune effector cells II. Transfer of experimental allergic encephalomyelitis with a subset of T lymphocytes. Eur. J. Immunol. 12: 453.
37. Richert, J. R., B. F. Driscoll, M.W. Kies, and E. C. Alvord, Jr. 1979. Adoptive transfer of experimental allergic encephalomyelitis incubation of rat spleen

cells with specific antigen. J. Immunol. 122. 494.

38. Killen, J. A., and R. H. Swanborg. 1982. Autoimmune effector cells. III. Role of adjuvant and accessory cells in the in vitro induction of autoimmune encephalomyelitis. J. Immunol. 129: 759.
39. Swanborg, R. H. 1983. Autoimmune effector cells. V. A monoclonal antibody specific for rat helper T lymphocytes inhibits adoptive transfer of autoimmune encephalomyelitis. J. Immunol. 130: 1503.
40. Brostoff, S. W., and D. W. Mason. 1986. The role of lymphocyte subpopulations in the transfer of rat EAE. J.Neuroimmunol. 10: 331.
41. Ortiz-Ortiz, L., and W. O. Weigle. 1982. Activation of effector cells in experimental allergic encephalomyelitis by interleukin 2 (IL-2). J. Immunol. 128: 1545.
42. White, R. A. H., D. W. Mason, A. F. Williams, G. Galfre, and C. Milstein. 1978. T lymphocyte heterogeneity in the rat: separation of functional subpopulations using a monoclonal antibody. J. Exp. Med. 148: 664.
43. Hayosh, N. S., L. L. Simon, and R. H. Swanborg. 1984. Autoimmune effector cells. VI. Transfer of experimental allergic encephalomyelitis with spleen cells activated in mixed lymphocyte cultures. J. Immunol. 133: 1943.
44. Ben-Nun, A., H. Wekerle, and I. R. Cohen. 1981. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. Eur. J. Immunol. 11: 185.
45. Ben-Nun, A., and I. R. Cohen. 1981. Vaccination against autoimmune encephalomyelitis (EAE): attenuated autoimmune T lymphocytes confer ressistance to induction of active EAE but not to EAE mediated by the intact T lymphocyte line. Eur. J. Immunol. 11: 949.
46. Naparstek, Y., A. Ben-Nun, J. Holoshitz, T. Reshef, A.

Froukel, M. Rosenberg, and I. R. Cohen. 1983. T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE): functional activation induces PNA receptors and accumulation in the brain and thymus of line cells. Eur. J. Immunol. 13: 418.

47. Steinman, L., J. T. Rosenbaum, S. Sriram, and H. O. McDevitt. 1981. In vivo effects of antibodies to immune response gene products: prevention of experimental allergic encephalomyelitis. Proc. Natl. Acad. Sci. U. S. A. 78: 7111.

48. Sriram, S., and L. Steinman. 1983. AntiI-A antibody suppresses active encephalomyelitis. J. Exp. Med. 158: 1362.

49. Trotter, J., S. Sriram, L. Rassenti, C-H. Jen Chou, R. B. Fritz, and L. Steinman. 1985. Characterization of T cell lines and clones from SJL/J and (BALB/cXSJL/J)F1 mice specific for myelin basic protein. J. Immunol. 134: 2322.

50. Vandenbark, A. A., P. Teal, and H. Offner. 1987. Activation of an encephalitogenic T lymphocyte line with a cell free supernatant containing basic protein and I region gene products. J. Immunol. 138: 452.

51. Alvord, Jr. E. C. 1970. Acute disseminated encephalomyelitis and "allergic" neuroencephalopathies. Handbook Clin. Neurol. 9: 500.

52. Kies, M. W. 1965. Chemical studies on an encephalitogenic protein from guinea pig brain. Ann. N. Y. Acad. Sci. 122: 161.

53. Nakao, A, W. J. Davis, and E. R. Einstein. 1966. Basic proteins from the acidic extract of bovine spinal cord. I. Isolation and characterization. Biochem. Biophys. Acta. 130: 163.

54. Nakao, A., W. J. Davis, and E. R. Einstein. 1966. Basic proteins from the acidic extract of bovine spinal cord. II. Encephalitogenic, immunologic and structural

interrelationships. Biochem. Biophys. Acta. 130: 171.

55. Raine, C. S. 1972. Viral infections of nerve tissue and their relevance to multiple sclerosis. In: Wolfgram, F., G. W. Ellison, J. G. Stevens, and J. M. Andrews (Eds.). Multiple sclerosis. Immunology, virology and ultrastructure. Academic Press. New York. p. 91.
56. Eylar, E. H., J. Caccam, and J. J. Jackson. 1970. Experimental allergic encephalomyelitis: synthesis of disease inducing site of the basic protein. Science 168: 1220.
57. Westall, F. C., A. B. Robinson, J. Caccam, J. Jackson, and E. H. Eylar. 1971. Essential chemical requirements for induction of allergic encephalomyelitis. Nature 229: 22.
58. Shapira, R., F. C-H. Chou, S. McKneally, E. Urban, and R. F. Kibler. 1971. Biological activity and synthesis of an encephalitogenic determinant. Science 173: 736.
59. Lees, M. B., and S. A. Paxan. 1974. Myelin protein from different regions of the central nervous system. J. Neurochem. 23: 825.
60. Hashim, G. A., D. D. Wood, and M. A. Moscarello. 1980. Myelin lipophilin-induced demyelinating disease of the central nervous system. Neurochem. Res. 5: 1137.
61. Vandenbark, A. A., T. Gill, and H. Offner, 1985. A myelin basic protein-specific T lymphocyte line that mediates experimental autoimmune encephalomyelitis. J. Immunol. 135: 223.
62. Pettinelli, C. B., R. B. Fritz, C. H. JenChou, and P. E. McFarlin. 1982. Encephalitogenic activity of guinea pig myelin basic protein in the SJL mouse. J. Immunol. 129: 1209.
63. Hashim, G. A., D. D. Wood, and M. A. Moscarello. 1980. Myelin-lipophilin-induced demyelinating disease of the central nervous system. Neurochem. Res. 5: 1137.
64. Cambi, F., M. B. Lees, R. M. Williams, and W. B. Macklin. 1983. Chronic experimental allergic

encephalomyelitis produced by bovine proteolipid apoprotein-immunological studies in rabbits. Ann. Neurol. 13: 303.

65. Yoshimura, T., T. Kunishita, K. Sakai, M. Endoh, T. Namikawa, and T. Tabira. 1985. Chronic experimental allergic encephalomyelitis in guinea pigs induced by proteolipid protein. J. Neurol. Sci. 69: 47.

66. Yamamura, T., T. Namikawa, M. Endoh, T. Kunishita, and T. Tabira. 1986. Experimental allergic encephalomyelitis induced by proteolipid apoprotein in lewis rats. J.Neuroimmunol. 12: 143.

67. Endoh, M, T. Tabira, T. Kunishiba, K. Sakai, T. Yamamura, and T. Taketomi. 1986. DM-20, a proteolipid apoprotein, is an encephalitogen of acute and relapsing autoimmune encephalomyelitis in mice. J. Immunol. 137: 3832.

68. Williams, R. M., M. B. Lees, F. Cambi, and W. B. Macklin. 1982. Chronic experimental allergic encephalomyelitis induced in rabbits with bovine white matter proteolipid apoprotein. J. Neuropath. Exp. Neurol. 41: 508.

69. Hosein, Z. Z., J. J. Gilbert, and G. H. Strejan. 1984. The role myelin lipids in experimental allergic encephalomyelitis. Part I. Influence on disease production by non-encephalitogenic doses of myelin basic protein. J. Neuroimmunol. 7: 163.

70. Linington, C., and H. Lassmann. 1987. Antibody responses in chronic replasing experimental allergic encephalomyelitis. Correlation of serum demyelination activity with antiboty titer to the myelin oligodendrocytes glycoprotein (MOG). J. Neuroimmunol. 17: 61.

71. Teuscher, C., E. P. Blankenhorn, and W. F. Hickey. 1987. Differential susceptibility to actively induced experimental allergic encephalomyelitis and experimental allergic orchitis among BALB/c subtrains.

Cell. Immunol. 110: 294.

72. Paterson, P. Y. 1966. Experimental allergic encephalomyelitis and autoimmune disease. Adv. Immunol. 5: 131.

73. Paterson, P. Y. 1971. The demyelinating disease: Clinical and experimental correlates. In Samter,M.(Ed): Immunological Disease, 2nd ed. Boston, Little Brown. p. 1289.

74. Paterson, P. Y. 1959. Organ-specific tissue damage induced by mammalian tissue adjuvant emulsions. In Lawrence,H.S. (Ed.): Cellular and humoral aspects of the hypersensitive states. New York. Harper & Row. pp. 467.

75. Pender, M. P. 1987. Demyelination and neurological signs in experimental allergic encephalomyelitis. J. Neuroimmunol. 15: 11.

76. Prineas, J., C. S. Raine, and H. Wisniewski. 1969. An ultra structural study of experimental demyelination and remyelination. III. Chronic experimental allergic encephalomyelitis in the central nervous system. Lab. Invest. 21: 472.

77. Brown, A., D. E. McFarlin, and C. S. Raine. 1982. Chronologic neuropathology of relapsing experimental allergic encephalomyelitis in the mouse. Lab. Invest. 46: 171.

78. Traugott, U., D. E. McFarlin, and C. S. Raine. 1986. Immunopathology of the lesion in chronic relapsing experimental autoimmune encephalomyelitis in the mouse. 99: 395.

79. Sriram, S., D. Solomon, R. V. Rouse, and L. Steinman. 1982. Identification of T cell subsets in mouse brain in experimental allergic encephalomyelitis. J. Immunol. 129: 1649.

80. Lampert, F., and P. Lampert. 1975. Multiple sclerosis (Eds.). Arch. Neurol. 32: 425.

81. Hickey, W. E., N. K. Gonatas, H. Kimura, and D. B.

Wilson. 1983. Identification and quantitation of T lymphocyte subsets found in the spinal cord of the Lewis rat during acute experimental allergic encephalomyelitis. J. Immunol. 131: 2805.

82. Cathala, F., P. Brown. 1972. The possible viral aetiology of disseminated sclerosis. J. Clin. Path. 25, Suppl.6.: 141

83. Norrby, E., H. Link, and J. E. Olsson. 1974. Measles virus antibodies in multiple sclerosis. Comparison of antibody titers in cerebrospinal fluid and serum. Arch. Neurol. 30: 285.

84. Jacobson, S., J. R. Richert, W. E. Biddson, A. Satinsky, R. J. Hartzman, and H. F. McFarland. 1984. Measles virus - specific T4+ human cytotoxic T cell clones are restricted by class II HLA antigens. J. Immunol. 133: 754.

85. Nathanson, M., and A. Miller. 1978. Epidemiology of multiple sclerosis: critique of evidence for a viral etiology. Amer. J. Epid. 107: 451.

86. Kurtzke, J. F., and K. Hyllested. 1986. Multiple sclerosis in the Faroe Islands. II. Clinical update, transmission, and the nature of MS. Neurology. 36: 307.

87. Sibley, W. A., C. R. Bamford, and K. Clark. 1985. Clinical viral infections and multiple sclerosis. Lancet i: 1313.

88. Salmi, A., M. Reunanen, and J. Ilonen. 1981. Possible viral etiology of multiple sclerosis. Proceedings of the 12th World Congress of Neurology. 568: 416.

89. Sissons, J. G. P., and L. K. Borysiewicz. 1985. Viral immunopathology. Bri. Med. Bull. 41: 34.

90. Watanabe, R., H. Wege, and v. ter Meulen. 1983. Adoptive transfer of EAE-like lesions from rats with coronavirus induced demyelinating encephalomyelitis. Nature 305: 150.

91. Sorensen, O., and S. Dales. 1986. The effect of T cell

functions on JHM virus infections of the rat CNS. Abstracts of the Satellite Symposium of the VIth International Congress of Immunology. p. 38.

92. Lipton, H. L. 1975. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. Infect.Immun. 11: 1147.
93. Rodriguez, M., L. R. Pease, and C. S. David. 1986. Immune mediated injury of virus infected oligodendrocytes. Immunol. Today 7: 359.
94. Rauch, H. C., I. N. Montgomery, W. Harb, and J. A. Benjamins. 1986. Chronic Theiler's virus infection: MBP appears in CSF and MBP antibody appears in serum. Abstracts of the Satellite Symposium of the VIth International Congress of Immunology. p. 37.
95. Atkins, G. J., B. J. Sheahan, and N. J. Dimmock. 1985. Semliki forest virus infection of mice: a model for genetic and molecular analysis of viral pathogenecity. J. Gen. Virol. 66: 395.
96. Kelly, W. R., W. F. Blackemore, and H. E. Webb. 1982. Demyelination induced in mice by avirulent semliki forest virus. II. An ultrastructural study of focal demyelination in the brain. Neuropathology and applied neurobiology. 8: 43.
97. Pathak, S., and H. E. Webb. 1978. An electron microscopic study of avirulent and virulent semliki forest virus in the brains of different ages of mice. J. Neurol.Sci. 39: 199.
98. Fazakerley, J. K., and H. E. Webb. 1987. Semliki forest virus - induced immune - mediated demyelination: adoptive transfer studies and viral persistence in nude mice. J. gen. Virol. 68: 377.
99. Massanari, R. M. 1981. Acceleration of experimental allergic encephalomyelitis in hamsters with antecedent virus infection. Clinical Immunol. Immunopath. 19: 457.
100. Massanari, R. M., P. Y. Paterson, and H. L. Lipton.

1979. Potentiation of experimental allergic encephalomyelitis in hamsters with persistent encephalitis due to measles virus. J. Infect. Dis. 139: 297.

101. Inada, T., and C. A. Mims. 1986. Infection of mice with lactic dehydrogenase virus prevents development of experimental allergic encephalomyelitis. J. Neuroimmunol. 11: 53.
102. Stroop, W. G., and M. A. Brinton. 1985. Enhancement of encephalomyeloradiculditis in mice sensitized with spinal cord tissue and infected with lactate dehydrogenase elevating virus. J. Neuroimmunol. 8: 79.
103. Hochberg, F. H., J. R. Lehrich, and B. G. W. Arnason. 1977. Herpes simplex virus infection and experimental allergic encephalomyelitis. Neurology 27: 584.
104. Rowson, K. E. K., and B. W. J. Mahy. 1985. Lactate dehydrogenase-elevating virus. J. gen. Virol. 66: 2297.
105. Webb, H. E., and J. K. Fazakerley. 1984. Can viral envelope glycolipids produce autoimmunity, with reference to the CNS and multiple sclerosis? Neuropath. Appl. Neurobiol. 10: 1.
106. Khalili-Shirazi, A., and H. E. Webb. 1986. Glycolipid cross reactivity between a demyelinating enveloped virus and CNS myelin. Implications for autoimmunity. Abstracts of the Satellite Symposium of the VIth International Congress of Immunology p. 34.
107. Gumbiner, C., P. Y. Paterson, G. P. Youmans, and A. S. Youmans. 1973. Adjuvanticity of microbacterial RNA and poly A:U for induction of experimental allergic encephalomyelitis in guinea pigs. J. Immunol. 110: 309.
108. Cone, R. E., and A. G. Johnson. 1971. Regulation of the immune system by synthetic polynucleotides. III. Action on antigen reactive cells of thymic origin. J. Exp. Med. 133: 665.

109. Inada, T., and C. A. Mims. 1984. Mouse Ia antigens are receptors for lactate degydrogenase virus. Nature 309: 59.

110. du Bay, H. G., and M. L. Johnson. 1966. Studies on the in vivo and in vitro multiplication of the LDH virus of mice. J. Exp. Med. 123: 985.

111. Porter, D. D., H. G. Porter, and B. B. Beerhake. 1968. Immunofluorescence assay for antigen and antibody in lactic dehydrogenase virus infection of mice. J. Immunol. 102: 431.

112. Inada, T., and C. A. Mims. 1985. Pattern of infection and selective loss of Ia positive cells in suckling and adult mice inoculated with lactic dehydrogenase virus. Arch. Virol. 86: 873.

113. Howard, R. J., A. L. Notkins, and S. E. Mergenhagen. 1969. Inhibition of cellular immune reactions in mice infected with lactic dehydrogenase virus. Nature 221: 873.

114. Michaelides, M. C., and E. S. Simms. 1977. Immune responses in mice infected with Lactic dehydrogenase virus II. Contact sensitization to PNFB and cheracterization of lymphoid cells during acute LDV infection. Cell. Immunol. 29: 285.

115. Mokhtarian, F., and P. Swoveland. 1987. Predisposition to EAE induction in resistant mice by prior infection with semliki forest virus. J. Immunol. 138: 3264.

116. Nash, A. A. 1985. Tolerance and suppression in virus diseases. Brit. Med. Bull. 41: 41.

117. Doherty, P. C. 1973. Quantitative studies of the inflammatory process in fatal viral meningoencephalitis. Am. J. Pathol. 73: 607.

118. Giulian, D., T. J. Baker, L. N. Shil, and L. B. Lachman. 1986. Interleukin 1 of the central nervous system is produced by ameboid microglia. J. Exp. Med. 164: 594.

119. Massa, P. T., R. Porries, and V. ter Meulen. 1986.

Viral particles induce Ia antigen expression on astrocytes. Nature 320: 543.

120. Fontana, A., U. Otz, A. L. DeWeek, and P. J. Grob. 1982. Glial cell stimulating factor (GSF): A new lymphokine. Part 2: Cellular sources and partial purification of human GSF. J. Neuroimmunol. 2: 73.
121. Fontana, A., A. Grieder, S. T. Arrenbrecht, and P. J. Grob. 1980. In vitro stimulation of glial cells by a lymphocyte produced factor. J. Neurol. Sci. 46: 55.
122. Durum, S. K., J. A. Schmidt, and J. J. Oppenheim. 1985. Interleukin-1: An immunological perspective. Annu. Rev. Immunol. 3: 263.
123. Merrill, J. E., S. Katsunai, C. Mohlstrom, F. Hofman, J. Groopman, and D. W. Golde. 1984. Proliferation of astroglial and oligodendroglial in response to human T cell derived factors. Science 224: 1428.
124. Benveniste, E. N., J. E. Merrill, S. E. Kaufman, D. W. Golde, and J. C. Gasson. 1985. Purification and characterization of a human T lymphocyte derived glial growth promoting factor. Proc. Natl. Acad. Sci. U. S. A. 82: 3930.
125. ter Meulen, V., P. T. Massa, V. G. Liebert, R. Dorries, and H. Wege. 1987. Autoimmunity to nervous tissue antigens after viral infections. J. Neuroimmunol. 16: 7.
126. Bottazzo, G. F., R. P. Borrell, and T. Hanafusa. 1983. Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. Lancet ii: 1115.
127. Bradish, C. J., K. Allner, and H. B. Maber. 1980. The virulence of original and derived strains of semliki froest virus for mice, guinea pigs and rabbits. J. Gen. Virol. 12: 141.
128. Pathak, S., and H. E. Webb. 1978. An electron-microscopic study of avirulent and virulent semliki forest virus in the brains of different ages of mice.

J. Neurol. Sci. 39: 199.

129. Kelly, W. R., W. F. Blackmore, S. Jagelman, and H. E. Webb. 1982. Demyelination induced in mice by avirulent semliki forest virus. II. An ultrastructural study of focal demyelination in the brain. Neuropathol. Appl. Neurobiol. 8: 43.

130. Pathak, S., S. J. Illavia, and H. E. Webb. 1983. The identification and role of cells involved in CNS demyelination in mice after SFV infection. An ultrastructural study. In: Immunology of Nervous System Infections. P. O. Behan, v. ter Meulen and F. C. Rose (Eds.). Amsterdam, Elsevier. p. 237.

131. Fazakerley, J. K., and H. E. Webb. 1987. Semliki forest virus induced immune mediated demyelination: adaptive transfer studies and viral persistence in nude mice. J. Gen. Virol. 68: 377.

132. Khalili-Shirazi, A., N. Gregson and H. E, Webb. 1986. Immunological relationship between a demyelinating RNA enveloped budding virus (Semliki forest) and brain glycolipids. J. Neurol. Sci. 76: 91.

133. Boere, W. A. M., M. Benaissa-Trouw, M. Harmsen, C. A. Kraaijeveld and H. Snippe. 1983. Neutralizing and non-neutralizing monoclonal antibodies to the E2 glycoprotein of semliki forest virus can protect mice from lethal encephalitis. J. Gen. Virol. 61: 405.

134. Kraaijeveld, C. A., B. Benaissa-Trouw, M. Harmsen, and H. Snippe. 1984. Delayed type hypersensitivity against semliki forest virus in mice: Local transfer of delayed type hypersensitivity with thioglycollate-induced peritoneal exudate cells. Int. Arch. Allergy Appl. Immunol. 73: 342.

135. Blackman, M. J., and A. G. Morris. 1984. Gamma IFN production and cytotoxicity of spleen cells from mice infecetd with semliki forest virus. J. Gen. Virol. 65: 955.

136. Rodda, S. J., and D. O. White. 1976. Cytotoxic

macrophages: a rapid nonspecific response to viral infection. J. Immunol. 117: 2067.

137. Jenkins, H. G., E. M. Tansey, F. Macofield, and H. Ikeda. 1988. Evidence for a T-cell related factor as the cause of demyelination in mice following semliki forest virus infection. Brain Research. 459: 145.

138. Tansey, E. M., and H. Ikeda. 1984. The relationship between axonal transport of protein and demyelination in the optic nerves of mice infected with semliki forest virus. Brain Research. 397: 9.

139. Hilfenhaus, J. 1976. Propagation of semliki forest virus in various human lymphoblastoid cell lines. J. Gen. Virol. 33: 539.

140. Adams, R. D. 1959. A comparison of the morphology of the human demyelinative disease and experimental " Allergic" encephalomyelitis, In: M. W. Kies, E. C. Alvord (Eds.), Springfied, Illinois, Charles C Thomas, p. 183.

141. Nathan, C. F., and Z. A. Cohn. 1984. Cellular components of inflammation: monocyte and macrophages. In: Textbook of Rheumatology, Chapter 10. W. Kelly, E.Harris, S. Ruddy and R.Hedge (Eds.), New York: W. B. Saunders, p. 144.

142. Cartwright, K. L., and D. G. Burke. 1970. Virus nucleic acids formed in chick embryo cells infected with Semliki forest virus. J. gen. Virol. 6: 231.

143. Garoff, H., K. Simons, and O. Renkonen. 1974. Isolation and characterization of the membrane polypeptides of Semliki forest virus. Virology. 61: 493.

144. Simons, K., H. Garoff, A. Helenius, and A. Ziemiecki. 1978. In Frontiers of physiochemical Biology. B. Pullman, editor. Acad. Press, Inc., New York. p.387.

145. Ziemeki, A., and H. Garoff. 1978. Subunit composition of the membrane glycoprotein complex of the Semliki forest virus. J. Mol. Biol. 122: 259.

146. Utermann, G., and K. Simons. 1974. Studies on the amphipathic nature of the membrane proteins in Semliki forest virus. J. Mol. Biol. 85: 569.

147. Kääriainen, L., and H. Söderlund. 1978. Structure and replication of alphaviruses. Curr. Top. Microbiol. Immunol. 82: 15.

148. Helenius, A., B. Morein, E. Fries, K. Simons, P. Robinson, V. Schirrmacher, C. Terhorst, and J. L. Strominger. 1978. Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki forest vurus. Proc. Natl. Acad. Sci. U. S. A. 75: 3846.

149. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki forest virus into BHK-21 cells. J. Cell Biol. 84: 404.

150. van der Groen, G., D. A. R. van der Berghe, and S. R. Pattyn. 1976. Interaction of mouse peritoneal macrophages with different arboviruses in vitro. J. gen. Virol. 34: 353.

151. Kraaijeveld, C. A., M. Harmsen, and B. K. Boutahar-Trouw. 1979. Cellular immunity against Semliki forest virus in mice. Infect. Immun. 23: 213.

152. Meinkoth, J., and S. I. T. Kennedy. 1980. Semliki forest virus persistence in mouse L929 cells. Virology. 100: 141.

153. Atkinson, T., A. D. T. Barrett, A. Mackenzie, and N. J. Dimmock. 1986. Persistence of virulent Semliki forest virus in mouse brain following co-inoculation with defective interferring particles. J. gen. Virol. 67: 1189.

154. Smithburn, K. C., and A. J. Haddow. 1944. Semliki forest virus. I. Isolation and pathogenic properties. J. Immunol. 49: 141.

155. McIntosh, B. M., C. Brookworth, and R. H. Kokernot. 1961. Isolation of Semliki forest virus from Aedes (Aedimorphus) argenteometatus (Theobald) collected in

Portugese east africa. Trans. Roy. Soc. Trop. Med. Hyg. 55: 192.

156. Smithburn, K. C. 1952. Studies on certain viruses isolated in the tropics of africa and south america. Immunological reactions as determined by cross - neutralization tests. J. Immunol. 68: 441.

157. Fleming, P. 1977. Age - dependent and strain - related differences of virulence of semliki forest virus in mice. J. gen. Virol. 37: 93.

158. Barrett, P. N., B. J. Sheahan, and G. J. Atkins. 1980. Isolation and preliminary characterization of Semliki forest virus mutants with altered virulence. J. gen. Virol. 49: 141.

159. Atkins, G. J., and B. J. Sheahan. 1981. Semliki forest virus neurovirulence mutants have altered cytopathogenicity for central nervous system cells. Infect. Immun. 36: 333.

160. Jagelman, S., A. J. Suckling, H. E. Webb, and E. T. W. Bowen. 1978. The pathogenesis of avirulent Semliki forest virus infections in nude mice. J. gen. Virol. 41: 599.

161. Fazakerley, J. K., S. Amor, and H. E. Webb. 1983. Reconstitution of Semliki forest virus infected mice, induces immune mediated pathological changes in the CNS. Clin. exp. Immunol. 52: 115.

162. Finter, N. B. 1966. Interferon as an antiviral agent in vivo: quantitative and temporal aspects of the protection of mice against Semliki forest virus. Brit. J. Exp. Pathol. 47: 361.

163. Morries, A., P. T. Tomkins, D. J. Maudsley, and M. Blackman. 1987. Infection of cultured murine brain cells by semliki forest virus: effects of Interferon α,β on viral replication, viral antigen display, major histocompatibility complex antigen display and lysis by cytotoxic T lymphocytes. J. gen. Virol. 68: 99.

164. Tomkins, P. T., G. A. Ward, and A. G. Morris. 1988. Role of interferon - gamma in T cell responses to Semliki forest virus infected brain cells. Immunology. 63: 355.

165. Allison, A. C. 1977. Autoimmune disease: Concepts of pathogenesis and control. In: Autoimmunity: Genetic, immunologic, virologic and clinical aspects. A. Tahl (Ed.). Acad. Press. New York. p. 91.

166. Bankhurst, A. D., G. Torrigiani, and A. C. Allison. 1973. Lymphocytes binding human thyroglobins in healthy people and its relevance to tolerance for autoantigens. Lancet i: 226.

167. Fujinami, R. and M. B. Oldstone. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. Science 230: 1043.

168. Nathanson, N., and A. Miller. 1978. Epidemiology of Multiple Sclerosis· Critique of the evidence for a viral etiology. Amer. J. Epi. 107: 451.

169. Lee, K. C., and M. Wong. 1980. Functional heterogeneity of culture growth bone marrow derived macrophages. I. Antigen presenting function. J. Immunol. 125: 86.

170. Lee, K. C., and M. Wong. 1982. Functional heterogeneity of culture growth bone marrow derived macrophages. II. Lymphokine stimulation of antigen presenting function. J. Immunol. 128: 2487.

171. Beller, D. I., and E. R. Unanue. 1982. Reciprocal regulation of macrophage and T cell function by way of soluble mediators. Lymphokines. 6: 25.

172. Simon, P. L., and W. F. Willoughby. 1982. Biochemical and biological characterization of rabbit interleukin-1 (IL-1). Lymphokines. 6: 47.

173. Waston, J., M. B. Frank, D. Mochizuki, and S. Gillis. 1982. The biochemistry and biology of interleukin-2. Lymphokines. 6: 95.

174. Dinarello, C. A. 1985. An update on human interleukin-1: from molecular biology to clinical relevance. J. Clin. Immunol. 5: 287.

175. Robb. R. J. 1984. Interleukin 2: The molecule and its function. Immunol. Today 5: 203.

176. Mizel, S. B. 1982. Interleukin 1 and cell activation. Immunol. Rev. 63: 51.

177. Fulbrigge, R. C., D. O. Chaplin, J. M. Kiely, and E. R. Unanue. 1987. Regulation of interleukin 1 gene expression by adherence and lipopolysaccharide. J. Immunol. 138: 3799.

178. Lemke, H., and H. Opitz. 1976. Function of 2 - mercaptoethanol as a macrophage substitute in the primary immune response in vitro. J. Immunol. 117: 388.

179. Goodman, M., and W. Weigle. 1977. Nonspecific activation of murine lymphocytes I. Proliferation and polyclonal activation induced by 2 - mercaptoethanol and alpha-thioglycerol. J. Exp. Med. 145: 473.

180. Howard, M., S. B. Mizel, L. Lachman, J. Ansel, B. Johnson, and W. E. Paul. 1983. Role of interleukin-1 in anti-immunoglobin induced B cell proliferation. J. Exp. Med. 157: 1529.

181. Goodmin, J., and J. Leuppens. 1983. Regulation of the immune response by prostaglandins. J. Clin. Immunol. 3: 295.

182. Knudsen, P. J., and T. B. Strom. 1985. Elevated intracellular cAMP levels inhibit interleukin-1 production by the tumor cell line U937. Brit. J. Rheumatol. 24: 65.

183. Kunkel, S. L., S. W. Chensue, and S. H. Phan. 1986. Prostaglandin as endogenous mediators of interleukin 1 production. J. Immunol. 136: 186.

184. Beller, D. I., and E. R. Unanue. 1981. Regulation of macrophage populations II. Synthesis and expression of Ia antigen by peritoneal exudate macrophages is a

transient event. J. Immunol. 126: 262.

185. Blackman, M. J., and A. G. Morris. 1984. Gamma interferon production and cytotoxicity of spleen cells from mice infected with Semliki forest virus. J. Gen. Virol. 65: 955.

186. Jenkins, H. G., E. M. Tansey, F. Macefield, and H. Ikeda. 1988. Evidence for a T cell related factor as the cause of demyelination in mice following semliki forest virus infection. Brain Research 459: 145.

187. Adams, D. O., and T. A. Hamilton. 1984. The cell biology of macrophage activation. Ann. Rev. Immunol. 2: 283.

188. Folch, J., M. B. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Biol. Chem. 266: 497.

189. Bizzozero, O., M. Bosio-Morene, J. M. Laoquini, E. F. Soro, and C. J. Gomez. 1982. Rapid purification of proteolipids from rat brain subcellular fractions by chromatography on a lipophilic dextran gel. J. Chromatograph. 227: 33.

190. Lees, M. B., and J. D. Sakura. 1979. Preparation of proteolipids. In Research Methods in Neurochemistry, N. Marks and R. Rodnight (Eds.). Plenum Press, New York, p. 354.

191. Gearing, A. J. H., A. Johnstone, and R. Thorpe. 1985. Production and assay of the interleukins. J. Immunol. Methods. 83: 1.

192. Weller, R. O. 1985. Pathology of Multiple Sclerosis. In: Mcalpine's Multiple Sclerosis. W. B. Mattews(Ed.). E & S Livingstone LTD. Edinburgh & London. p. 301.

193. Batchelor, F. R. 1985. Immunological and genetic aspects of multiple sclerosis. In: Mclpine's Multiple Sclerosis. W. B. Mattews (Ed.). E & S Livingstone Ltd. Edinburgh & London. p. 281.

194. Kurtzke, J. F., and K. Hyllested. 1986. Multiple

sclerosis in the Faroe Islands. II. Clinical update, transmission, and the nature of MS. Neurology. 36: 307.

195. Norrby, E., H. Link, and J. E. Olsson. 1974. Measles virus antibodies in multiple sclerosis. Arch. Neurol. 30: 285.

196. Adams, J. M., and D. T. Imagewa. 1962. Measles antibodies in multiple sclerosis. Proc. Soc. Exp. Biol. Med. iii: 562.

197. Brody, J. A. 1972. Measles antibody titers of multiple sclerosis patients and their siblings. Neurology. 22: 492.

198. Johnson, R. T. 1975. The possible viral etiology of multiple sclerosis. Adv. Neurol. 13: 1-46.

199. Brosnan, C. F., K. Selmaj, and C. S. Raine. 1988. Hypothesis: A role for tumor necrosis factor in immune mediated demyelination and its relevance to multiple sclerosis. J. Neuroimmunol. 18: 87.

200. Sissons, J. G., and L. K. Borysiewicz. 1985. Viral immunopathology. Brit. Med. Bull. 41: 34.

201. Pathak, S., and H. E. Webb. 1988. An electron microscopical study of the replication of avirulent Semliki Forest virus in the retine of mice. J. Neurol. Sci. 85: 87.

202. Khalili-Shirazi, A., N. Gregson, and H. E. Webb. 1988. Immunocytochemical evidence for Semliki Forest virus antigen persistence in mouse brain. J. Neurol. Sci. 85: 17.

203. Simons, K., H. Garoff, and A. Helenius. 1988. How an animal virus gets into and out of its host cell. Sci. Amer. 246: 58.

204. Sedgwick, J., S. Brostoff, and D. Mason. 1987. Experimental allergic encephalomyelitis in the absence of a classical delayed-type hypersensitivity reaction. J. Exp. Med. 165: 1058.

205. Sy, M-S., M. Tsurufuji, R. Finberg, and B. Benacerraf.

1983. Effect of vesicular stomatitis virus (VSV) infection on the development and regulation of T cell mediated immune responses. J. Immunol. 131: 30.

206: Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin. 1983. Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. 158: 670.

207. Heremans, H., R. Dijkmans, H. Sobis, F. Vandekerckhove, and A. Billiau. 1987. Regulation by interferons of the local inflammatory responses to lipopolysaccharide. J. Immunol. 138: 4175.

208. Landolfo, S., M. Gariglio, G. Gribaudo, C. Jemma, M. Giovarelli, and G. Cavallo. 1988. Interferon-γ is not an antiviral , but a growth-promoting factor for T lymphocytes. Eur. J. Immunol. 18: 503.

209. Lindholm, D., R. Heumann, M. Meyer, and H. Thoenen. 1987. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. Nature 330: 658.

210. Dinarello. 1988. Biology of interleukin 1. FASEB. J. 2: 108.

211. Miyajima, A., S. Miyatake, J. Schreurs, J. De Vries, N. Arai, T. Yokota, and A. Keu-Ichi. 1988. Coordinate regulation of immune and inflammatory responses by T cell derived lymphokines. FASEB. J. 2: 2462.

212. Baglioni, C., and P. A. Maroney. 1980. Mechanism of action of human interferons. Induction of 2' 5' - oligo(A) polymerase. J. Biol. Chem. 255: 8390.

213. Lengyel, P., 1983. Biochemistry of interferons and their actions. Ann. Rev. Biochem. 51: 251.

214. Sen, G. C. 1984. Biochemical pathways in interferon-action. Pharmacol. Ther. 24: 235.

215. Billiau, A., H. Hereman, F. Vanderckerckhove, R. Dijkmans, H. Sobis, E. Meulepes, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis

in mice by antibodies against IFN-γ. J. Immunol. 140: 1506.

216. Stewart, C. C., H. Lin, C. Adles. 1975. Proliferation and colony forming ability of peritoneal exudate cells in liquid culture. J. Exp. Med. 141: 1114.

217. Abreu, S. 1982. Suppression of experimental allergic encephalomyelitis by interferon. Immunol. Commun. 11: 1.

218. Parham, P. 1988. Intolerable secretion in tolerant transgenic mice. Nature 333: 500.

219. Cherwinski, H., J. Schumacher, K. Brown, and T. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. 166: 1229.

220. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348.

221. Meuer, S. C., R. E. Hussey, D. A. Cautrell, J. C. Hodgdon, S. F. Schlossman, K. A. Smith, and E. L. Reinherz. 1984. Triggering of the T3-Ti antigen-receptor complex results in clonal T cell proliferation through an interleukin 2 dependent autocrine pathway. Proc. Natl. Acad. Sci. USA 81: 1509.

222. Lepe-Zuniga, J. L., P. W. Mansell, and E. M. Hersh. 1987. Idiopathic production of interleukin-1 in acquired immune deficiency syndrome. J. Clin. Microbiol. 25: 1695.

223. Knudsen, P. J., and T. B. Strom. 1985. Elevated intracellular cAMP levels inhibit interleukin-1 production by the tumor cell line U937. Brit. J. Rheumatol. 24: 65.

224. Rodgers, B. L., D. M. Scott, J. Mundin, and J. G. P. Sissons. 1985. Monocyte-derived inhibitor of interleukin-1 induced by human cytomegalovirus. J. Virol. 55: 527.

225. Brown, K., and D. L. Rosenstreich. 1987. Mechanism of action of a human interleukin 1 inhibitor. Cell. Immunol. 105: 45.

226. Wu, L-X, J. Ilonen, M. J. Mäkelä, R. Salonen, R. Marusyk, and A. A. Salmi. 1988. Impaired interleukin 1 and interleukin 2 production following in vitro abortive infection of murine spleen mononuclear cells by Semliki Forest virus. Cell. Immunol. 116: 112.

227. Joseph, B. S., P. W. Lampert, and M. B. A. Oldstone. 1975. Replication and persistence of measles virus in defined subpopulations of human leukocytes. J. Virol. 16: 1638.

228. Hyypiä, T., P. Korkiamäki, and R. Vainionpää. 1986. Replication of measles virus in human lymphocytes. J. Exp. Med. 161: 1261.

229. Goodman, M., and W. Weigle. 1977. Nonspecific activation of murine lymphocytes. I. Proliferation and polyclonal activation induced by 2-mercaptoethanol and alpha-thioglycerol. J. Exp. Med. 145: 437.

230. Beller, D. I., and E. R. Unanue. 1981. Regulation of macrophage production. II. Synthesis and expression of Ia antigen by peritoneal exudate macrophages is a transient event. J. Immunol. 1: 31

231. Mahoney, E. M., A. L. Hanmill, W. A. Scott, and Z. A. Cohn. 1977. Response of endocytosis to altered fatty acyl composition of macrophage phospholipids. Proc. Natl. Acad. Sci. USA 74: 4895.

232. Stahl, P., P. H. Schlesinger, E. Sigardson, J. S. Rodman, and Y. C. Lee. 1980. Receptor mediated pinocytosis of mannose glycoconjugates by macrophages: Characterization and evidence for receptor recycling. Cell. 19: 207.

233. Klatzmann, D., F. B. Sinoussi, M. T. Nugeyer, C. Dauguct, E. Vilmer, C. Griscelli, F. B. Vezinet, C. Rouzioux, J. C. Gluckman, J. C. Chermam, and L. Montagnier. 1984. Selective tropism of lymphoadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 225: 59.

234. Morahan, P. S., J. Onnor, and K. R. Leary. 1985. Viruses and the versatile macrophages. Brit. Med. Bull. 41: 15.

235. Kumar, A., and U. Lindberg. 1972. Characterization of messenger ribonucleoprotein and messenger RNA from KB cells. Proc. Natl. Acad. Sci. USA. 69: 681.

236. Glickman, J. M., J. G. Howe, and J. A. Steitz. 1988. Structural analyses of EBER1 and EBER2 ribonucleoprotein particles in Epstein-Barr virus infected cells. J. Virol. 62: 902.

237. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

238. Lutler, D. F., and H. Garoff. 1986. Mutants of the membrane binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. J. Cell. Biol. 102: 889.

239. Garoff, H., A. M. Frischauf, K. Simons, H. Lehrach, and J. Delius. 1980. Nucleotide sequence of cDNA coding for Semliki Forest virus membrane glycoproteins. Nature 288: 236.

240. Kuchroo, V. K., M. Minami, B. Diamond, and M. E. Dorf. 1988. Functional analysis of cloned macrophage hybridomas. VI. Differential ability to induce immunity or suppression. J. Immunol. 141: 10.

241. Delovitch, T. L., J. W. Semple, and M. L. Phillips. 1988. Influence of antigen processing on immune responsiveness. Immunol. Today 9: 216.

242. Koh, C-S, and P. Y. Paterson. 1987. Suppression of clinical signs of cell-transferred experimental

allergic encephalomyelitis and altered cerebrovascular permeability in lewis rats treated with plasminogen activator inhibitor. Cell. Immunol. 107: 52.

243. L. W-L. Chui, R. Vainionpää, R. G. Marusyk, A. Salmi, and E. Norrby. 1986. Nuclear accumulation of measles virus nucleoprotein associated with a temperature-sensitive mutant. J. Gen. Virol. 67: 2153.

244. Bottomly, K. 1988. A functional dichotomy in CD4+ T lymphocytes. Immunol. Today 9: 268.

245. Gonatas, N. K., M. I. Greene, and B. H. Waksman. 1986. Genetic and molecular aspects of demyelination. Immunol. Today 7: 121.

246. Ilonen, J., R. Salonen, R. Marusyk, and A. Salmi. 1988. Measles virus strain-dependent variation in outcome of infection of human blood mononuclear cells. J. gen. Virol. 69: 247.