ANTIBODY FORMATION AND IMMUNOMODULATION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS AND MULTIPLE SCLEROSIS

The studies described in this thesis were performed at the department of Immunology and Medical Microbiology (head: Prof. Dr. E.H.J.H.M. Claassen) of the TNO-Medical Biological Laboratory in Rijswijk, The Netherlands.

The research project was financially supported by a grant (MS89-50) of the foundation "Stichting Vrienden MS Research".

The publication of this thesis was sponsored by:
- The foundation "Stichting Vrienden MS Research"
- TNO-Prins Maurits Laboratory, Research Group Chemical Toxicology

Cover design: P. Gerritse
Printing: Optima druk, Molenaarsgraaf
ISBN: 90-9008490-8

ANTIBODY FORMATION AND IMMUNOMODULATION IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS AND MULTIPLE SCLEROSIS

ANTILICHAAMVORMING EN MODULATIE VAN
HET IMMUUNSYSTEEM IN
EXPERIMENTELE ALLERGISCHE ENCEPHALOMYELITIS
EN MULTIPLE SCLEROSE

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
donderdag 28 september 1995 om 13.30 uur.

door
Koen Gerritse
geboren te Rotterdam

PROMOTIE-COMMISSIE:

Promotor:	Prof. Dr. E.H.J.H.M. Claassen
Co-promotor:	Dr. W.J.A. Boersma
Overige leden:	Prof. Dr. J.J. Haaijman Prof. Dr. A.D.M.E. Osterhaus Prof. Dr. F.G.A. v.d. Meché

Voor Karin

CONTENTS

Synopsis		8
Chapter 1	**Introduction**	11
1.1	General	12
1.2	The central nervous system	12
1.3	Diagnosis and clinical aspects	13
1.4	Histo-pathology	16
1.5	Therapy	17
1.6	Etiology	17
1.6.1	Geographical distribution and genetic factors	18
1.6.2	Immunological factors	18
1.6.2.1	MHC	19
1.6.2.2	T-cell reactivity	20
1.6.2.3	B-cell reactivity	22
1.6.2.4	Molecular mimicry	23
1.6.3	Infectious agents	24
1.7	Animal models	24
1.8	Outline of the thesis	28
Chapter 2	***In situ* B-cell responses in MS and EAE**	31
2.1	Introduction to *in situ* B-cell responses in MS and EAE	32
2.2	New immuno-enzyme-cytochemical stainings for the *in situ* detection of epitope specificity and isotype of antibody forming B-cells in experimental and natural (auto)immune responses in animals and man **J Immunol Methods 150:207-216, 1992**	36
2.3	Conjugate formation in urea: Coupling of insoluble peptides to alkaline phosphatase for ELISA and *in situ* detection of antibody forming cells **J Histochem Cytochem 39:987-992, 1991**	46
2.4	*In situ* detection of self reactive epitope specific antibody forming cells in the central nervous system of EAE rhesus monkey **EOS J Immunol Immunopharmacol 13:63-65, 1993**	58
2.5	The involvement of specific anti myelin basic protein antibody forming cells in multiple sclerosis immunopathology **J Neuroimmunol 49:153-159, 1994**	64
2.6	Discussion: Significance of autoantigen specific antibody forming cells in multiple sclerosis	74

Chapter 3	**Modulation of EAE by blockade of CD40-CD40-ligand interactions**	79
3.1	Functional and histological evidence for CD40-CD40-ligand interactions in multiple sclerosis **Submitted for publication**	80
Chapter 4	**Oral delivery of MS-autoantigens by *Lactobacillus* to induce tolerance**	97
4.1	The use of *Lactobacillus* as carrier for the oral delivery of antigens	98
4.2	Oral administration of TNP-*Lactobacillus* conjugates in mice: A model for evaluation of mucosal- and systemic immune responses and memory formation elicited by transformed lactobacilli **Res Microbiol 141:955-962, 1990**	106
4.3	Mucosal immune responses and systemic immunological memory after oral administration of trinitrophenyl *Lactobacillus* conjugates in mice **Lymphatic Tissues and *in vivo* Immune Responses 84:497-504, 1991**	114
4.4	*Lactobacillus* as a vector for oral delivery of antigens: The role of intrinsic adjuvanticity in modulation of immune responses **Submitted for publication**	124
Chapter 5	**General discussion**	139
References		150
Summary/Samenvatting		178
Abbreviations		184
Publications		186
Curriculum Vitae		188
Dankwoord		190

SYNOPSIS

Multiple sclerosis (MS) is a chronic demyelinating disease characterized by perivascular accumulation of inflammatory cells, resulting in numerous (multiple) plaques (sclerosis), in the central nervous system (CNS).

Despite ample research efforts both etiology and pathogenicity of the disease remain largely unknown. Due to the complexity of clinical manifestations the unequivocal diagnosis of MS is difficult to make. It is generally accepted that the effector phase of the disease depends on an autoimmune reaction. Notwithstanding the fact that oligoclonal antibodies can be found in the cerebrospinal fluid during disease, the role of antibodies in MS is still unclear. In order to investigate the involvement of autoantigen specific antibodies in the pathology of MS, we performed a study to detect antigen specific B-cells (AFCs) in CNS tissues of MS patients. Using a new immunohistochemical technique we have revealed that a significant part of the AFCs localized in MS brain is specific for myelin basic protein (MBP). This provides further evidence for the local involvement of anti-MBP antibodies in the pathogenesis of MS. Production of antibodies by B-cells is critically dependent on T-cell help. It has been shown that interactions between the gp39 T-cell marker and its CD40 ligand on B-cells are essential for B-cell activation. In our experiments we have revealed gp39 positive T-cells in the same affected CNS tissue areas (plaques) in which we have detected the MBP specific AFC. The presence of both $gp39^+$ Th-cells and MBP specific B-cells in plaques of MS patients CNS tissues indicates that autoantigen specific B-cells can be activated directly within the CNS.

The relevance, of this new observation in human tissue, was further established when it was shown that blockade of the gp39/CD40 interaction in EAE mice, resulted in dramatic suppression of the disease. Both from studies in mice and man it became clear that *in vivo* blockade, of gp39/CD40 interactions, has a significant potential in the treatment of MS and other autoimmune diseases. An alternative approach to induce long term antigen specific suppression is the induction of oral tolerance. Tolerance can be induced by oral administration of large doses of putative auto-antigens. Live non-pathogenic microorganisms, transformed to produce antigens, are useful for oral delivery of antigens as they have many advantages over conventional feeding. Here we show that oral administration of genetically modified lactobacilli, which secrete antigen, leads to the induction of tolerance. In contrast, in a parallel line of research we show that oral administration of lactobacilli which express antigens on the outer membrane leads to induction of immunological memory and can thus be employed for vaccination against infectious diseases. Whether the immune system is stimulated or suppressed not only depends on the way the (auto-)antigen is presented (i.e. secreted or surface bound) but also on the intrinsic adjuvant properties of the *Lactobacillus* strain used. This indicates that modulation of immune responses can lead to suppression of disease in animals and possibly in MS.

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 General

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). It is widely assumed that the pathology is a result of autoimmune responses directed against CNS antigens, possibly triggered by an environmental factor, in a genetically susceptible individual.

This thesis describes investigations concerning the role of antibodies and antibody forming cells in immunopathogenesis and immunomodulation in MS. In this chapter we introduce some basic understandings with regard to the central nervous system, the pathogenesis and etiology of MS and the presently best available animal model for MS, experimental autoimmune encephalomyelitis (EAE). The aim of the study and experiments are described in "Outline of the thesis" (1.8).

1.2 The central nervous system

Most body functions are actively controlled by the CNS. The CNS receives information from sensory organs. Upon processing of this information, the CNS generates and controls body responses. In addition, the information may also be stored in the CNS for future use. The information to and from the CNS is transported via the peripheral nervous system. The major compartments within the CNS are the brain and the spinal cord, which are surrounded by the cerebrospinal fluid (CSF) and protected by the skull and vertebral column, respectively. CNS tissues are a complex of nerve cells (neurons), supporting cells (glia cells or neuroglia) and blood vessels. Three basic types of glia cells can be identified: astrocytes and microglia, both contributing to the structure and function of nervous tissue, and oligodendrocytes. Nerve fibres are protected by an isolating myelin sheath. The myelin around the axons in the CNS, is formed by layers of oligodendrocyte cell cytoplasm and membranes, which are wrapped many times around one or more axons (figure 1). The myelin sheaths in the peripheral nervous system are formed, in a similar way, by Schwann cells (England and Wakely, 1991).

Demyelination is associated with an impaired conductivity, which leads to a variety of neurological signs and symptoms. Several demyelinating diseases are known of both the peripheral nervous system, e.g. Guillain Barré syndrome, and CNS, e.g. acute demyelinating encephalomyelitis, subacute sclerosing pan-encephalitis. MS is characterized by multi-focal demyelination of CNS white matter, accompanied with perivascular infiltrates of mononuclear cells.

Since demyelination in MS-patients is restricted to the CNS, it is possible that

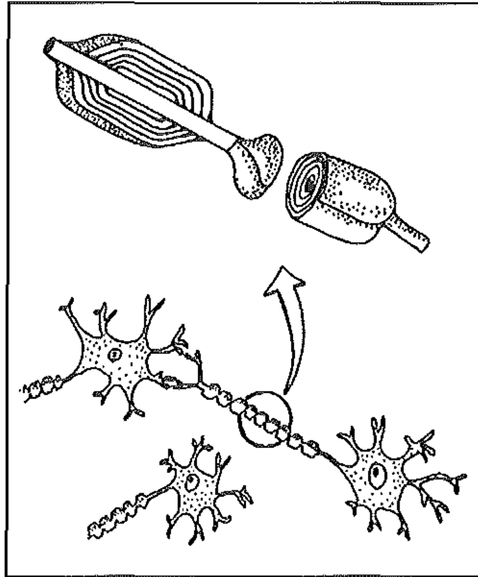


Figure 1
The Myelin sheaths are formed by thin layers of oligodendrocyte cytoplasm and membranes which are wrapped around the axons. Artistic impression.

demyelination is associated with a defect concerning the CNS specific oligodendrocytes. Mutations within the gene coding for proteolipid protein (PLP). may cause hypomyelination and premature death of oligodendrocytes as was observed in the jimpy mouse model and patients with Pelizaeus-Merzbacher disease (Vermeesch et al., 1990; Pratt et al., 1991). PLP is one of the major proteins in CNS myelin which is considered to be synthesized exclusively by oligodendrocytes (Lees and Brostoff, 1984; Macklin, 1988).

1.3 Diagnosis and clinical aspects

Dependent on the severity and localization of the demyelinated areas (plaques or scars) in the CNS, a variety of clinical signs may become manifest in MS. Neurological signs and symptoms include fatigue, weakness or paralysis, disturbed gait, balance abnormalities, vision abnormalities (double vision, partial or complete blindness, nystagmus), bladder and bowel difficulties, sexual difficulties and psychiatric and cognitive defects (Ron and Feinstein, 1992; Matthews, 1991). The sequence of appearance and severity of clinical signs and symptoms, is highly diverse. As a consequence, it is very difficult to diagnose MS in an early stage.

Several sets of criteria have been developed to score the level of disability of patients in order to support the diagnosis of MS. Allison and Millar (1954)

developed criteria for MS based on neurological signs and symptoms. Using these criteria patients may be classified as A) probable MS, B) early probable or latent MS and C) possible MS. The sets of criteria developed by Schumacher and coworkers (1965) requires for a definite MS diagnosis that neurological signs and symptoms are attributed to more than one lesion and neurological signs and symptoms have become manifest in two or more separate periods. Most other developed criteria are variants to the above mentioned sets. The currently most general used criteria, which relies on both neurological signs and symptoms and laboratory data, are those developed by Posner et al (1983). These criteria distinguish patients into: A) clinically definite MS (2 subcategories), B) laboratory supported definite MS (3 subcategories), C) clinically probable MS (3 subcategories) and D) laboratory supported probable MS. As there is no single specific test to diagnose MS, in general a combination of clinical and laboratory tests is used for diagnostic purposes.

By means of computerized tomography (CT), lesions can be identified, in particular when CT is combined with contrast enhancement (Aita et al., 1978). An other technique to identify plaques is magnetic resonance imaging (MRI) (figure 2). Small lesions (10-12 mm^2) can still be identified (Young et al., 1981). MRI presently is the most sensitive technique to confirm diagnosis and course of the disease (Uhlenbrock et al., 1988). In addition, MRI scanning allows quantitative measurements of lesions before and after treatment. Gadolinium diethylenetriamine acetic acid enhancement, increases the sensitivity of MRI and makes it possible to distinguish between early inflammation and chronic lesions (Thompson et al., 1989). However, using MRI many silent lesions were identified that often fail to correlate with clinical signs (Ormerod et al., 1987; Oger et al., 1987; Willoughby et al., 1989).

Electrophysiological studies to investigate the visual, auditory and somatic sensory pathways can provide evidence on lesions in the CNS (McDonald and Halliday, 1977). In addition, electrophysiological studies may be useful in evaluating the progression of the disease.

With regard to the course of disease in MS, four major phases in the disease can be distinguished (figure 3).

- A) Relapsing remitting (RR) MS; In most MS-patients the disease starts with periods in which some of the typical clinical signs become manifest (exacerbations), alternating with periods in which clinical signs are absent (remissions). The appearance of the exacerbations is unpredictable and the remissions vary in duration. In the early stages of the disease the remissions are usually associated with nearly complete restoration of normal neurological functions. This phase of disease is designated as relapsing remitting MS.
- B) Secondary progressive MS; In one third of the MS patients the RR phase of disease is followed by a second more progressive phase in the disease. This phase of disease is characterized by the development of an increasing disability over at least six months.

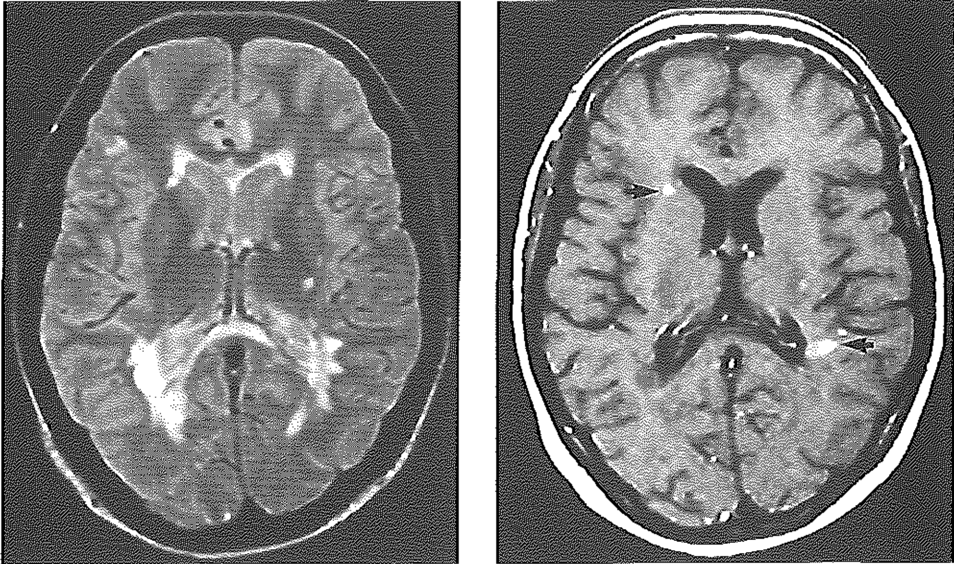


Figure 2
Magnetic resonance images of MS patient brain (horizontal plane), showing periventricular plaques (left) and several focal plaques (right) (arrows). Courtesy of Dr. F. Barkhof, VU hospital, Amsterdam, The Netherlands.

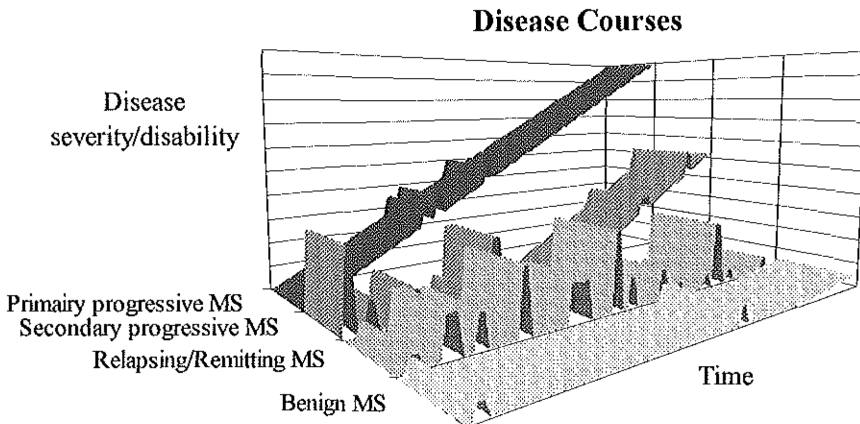


Figure 3
The clinical course of MS exhibits a number of patterns. Four main clinical subgroups can be distinguished:
- Benign MS, minimal or no disability after 10 years of disease duration.
- Relapsing remitting MS, a history of relapses and remissions without progressive deterioration.
- Primary progressive MS, progressive from the onset.
- Secondary progressive MS, after an initially relapsing-remitting course, progressive deterioration for at least six months, with or without superimposed relapses.

- C) Benign MS; A relative mild form of MS is benign MS, in which the patients can remain relatively asymptomatic for many years. The condition of RR-MS patients, which have an expanded disability status scale score of 3 or less, according to the criteria developed by Kurtzke (1983a), after a period of at least 10 years is defined as benign MS.
- D) Primary progressive MS; A minor part of MS patients will develop, over a few weeks or months, a progressive course of disease from the onset of disease without relapses or remissions, this course of disease is called primary progressive MS. Primary progressive MS can be terminal within a few months.

In most patients the onset of the disease lies between their 20th and 40th year of age. The first appearance of typical MS clinical signs or symptoms in children or adults over 55 year of age has been reported occasionally (Gall et al., 1958; Low and Carter, 1956; Ghezzi et al., 1978). In The Netherlands the prevalence of MS is 60 cases per 100.000 inhabitants and the yearly incidence is 1.9 (de Graaf et al., 1988). In women, MS is diagnosed approximately twice as often as compared to the frequency of diagnoses of MS in men. The average age at which MS patients decease is 63 year. In most cases of MS the cause of death is found in accompanying secondary diseases, mainly infections.

1.4 Histo-pathology

MS is primarily a demyelinating disease, in which the myelin sheaths are selectively damaged leaving the axons relatively unaffected. The colour of the demyelinated areas or plaques correlates with the age of plaques. New plaques are pink whereas old plaques are grey (Adams and Sidman., 1968). Plaques can be found throughout the complete CNS. Cortical and peri-ventricular plaques have been found in the majority of MS patients (Lumsden, 1970). Two types of plaques can be distinguished: acute plaques and chronic plaques (Adams, 1983). Acute plaques are not clearly demarcated and they contain T-cells, B-cells, macrophages and myelin fragments which is indicative for demyelination activity (Traugott and Raine, 1982; Traugott et al., 1983; Lumsden, 1970). Periventricular cuffs, accumulations of lymphocytes around the ventricles, are often found in the vicinity of active plaques and suggest migration of lymphocytes from the ventricles into the CNS tissues. Re-myelination and oligodendrocyte proliferation is observed in the majority of acute plaques (Prineas, 1985). Chronic plaques contain hypertrophic astrocytes, microglia and plasma cells, producing antibodies of unknown specificity. Axons without a myelin sheath can also be found in chronic plaques. Re-myelination occurs to a limited extent in chronic plaques, however the newly formed myelin sheaths are thinner (Prineas and Connell, 1979).

1.5 Therapy

The factors that lead to development of MS have not been identified yet. Based on the premise that MS is mediated by an aberrant immune response, both immuno-suppressive and immunopotentiating therapies have been applied to MS patients. However, one can presume that therapy affecting symptomatology in the peripheral immune system only, like plasmapheresis and plasma exchange, will have no or little effect on the continuity of immune processes within the CNS. In addition, most drugs with immunosuppressive or immunopotentiating effects, do not cross the blood brain barrier when applied systemically.

A temporarily stabilizing effect on chronic progressive MS was observed with intensive immunosuppression based on the administration of cyclophosphamide or total lymphoid irradiation (Hauser et al., 1983; Weiner et al., 1985; Cook et al., 1986). A 10-14 day treatment of high doses cyclophosphamide and adrenocorticotropic hormone (ACTH) stabilized progressive MS for an average of 18 months (Carter et al., 1986). In MS patients treated with a combination of cyclophosphamide and ACTH a decrease was demonstrated in spontaneous T-cell proliferation and changes in certain T-cell populations (Hafler et al., 1991). Chronic administration of azathioprine is only effective in MS patients with mild, or recent onset of disease (Caputo et al., 1987). Other forms of therapy which are under study or have been studied are: the administration of monoclonal antibodies directed against putative reactive T-cells (Hafler and Weiner, 1988; Hafler et al., 1986); induction of tolerance by oral administration of myelin basic protein (MBP) (Weiner et al., 1993); the induction of an immune response against the antigen receptor of autoantigen reactive T-cells by the administration of peptides analogous to sequences of the T-cell receptor (Vandenbark et al., 1993); immunosuppression by cyclosporin A treatment (Calder et al., 1987); immunopotentiation especially by interferons (Panitsch et al., 1985; 1987; Knobler et al., 1984; Greenstein et al., 1987; Rice et al., 1985); acupuncture; diets (Swank, 1991) and drugs which enhance remyelination or improve the axonal conductivity (Tourtelotte and Baumhefner, 1983).

Recently, Interferon-β was approved in the United States for prophylactic treatment of relapsing-remitting MS (Arnason and Reder, 1994). However, most of the mentioned therapies have proved to be of limited value only. The administration of gamma-interferon to MS patients produces even the opposite effect (Panitsch et al., 1987).

1.6 Etiology

Despite intensive clinical and experimental research, the etiology of MS is still unknown. Several factors have been hypothesized to be involved in the induction and/or enhancement of the disease. A) Geographic location; The involvement of an

environmental factor is supported by the geographical distribution of MS (Kurtzke, 1983b). B) Genetic factors; The susceptibility to MS is linked to certain genetic backgrounds. C) (Auto)immunity; The putative commitment of the immune system and autoimmune processes particular in MS is largely based on the resemblance of MS with the animal model, experimental autoimmune encephalomyelitis. D) Infection; The occurrence of MS epidemics points to an infectious agent as the environmental factor. Although none of the factors mentioned in itself can be regarded as the causative factor of MS, it is still possible that a combination of the factors, i.e. the combination of the environmental factors in a genetic and/or immunological susceptible subject, may lead to the development of MS. In the next section the involvement of the factors in the etiology of MS will be discussed.

1.6.1 Geographical distribution and genetic factors

The geographic distribution of MS suggests the influence of an environmental and/or a genetic factor in the initiation of the disease. Regions of high prevalence are found in areas with a moderate climate between the 45 and 65 latitudes (Kurtzke, 1980a; 1983b) (figure 4). The prevalence of MS in Asia and Africa is approximately a factor 10 lower as compared to the prevalence in The Netherlands. However, immigrants from regions with a low prevalence do not have a higher risk of developing MS when they move to areas with a high prevalence, unless they are 15 years of age or less at the time of migration (Alter et al., 1978).

There are clear genetic influences on the susceptibility to MS. In studies of familial MS it was revealed that in 26% of monozygotic twin pairs both twins were affected, whereas MS was diagnosed in only 2.3% in both twins of dizygotic twin pairs (Ebers, 1986). Furthermore, the risk of developing MS is slightly increased in first and second degree relatives of MS patients (McAlpine et al., 1972). However, the increased incidence of MS in relatives and dizygotic twins may also be due to a common environmental factor shared by family members and twins respectively.

1.6.2 Immunological factors

In a normally functioning organism, the immune system is quite capable of recognizing and effectively dealing with foreign antigens leaving the host cells and tissues unaffected. Moreover, the immune system does not react with foreign functional antigens, like food constituents. However, sometimes this delicate balance is disturbed and the immune system fails to maintain this so called self tolerance, resulting in destruction of (self) cells and tissues. Although many distinctive cells of the immune system are involved in autoimmunity, only B and T-cells are involved in the actual recognition and discrimination between self- and nonself antigens. B-cells can recognize unprocessed (auto)antigen directly by their antigen receptors, the membrane bound antibodies. In contrast T-cells can only recognize processed

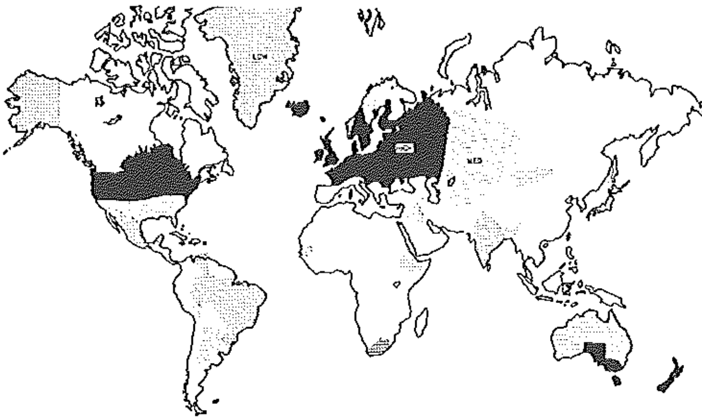


Figure 4
The global prevalence of multiple sclerosis as of 1980. Areas with high, medium and low frequencies of MS are indicated in black, dark grey and light grey respectively. No data are available on non-coloured areas. From Dr. J.F. Kurtzke, Acta Neurol Scand 62:65, 1980. Reproduced with permission of Acta Neurologica Scandinavica.

(auto)antigens which are presented to the T-cell receptor (TCR) by the major histocompatibility complex (MHC) expressed on antigen presenting cells.

1.6.2.1 MHC

Like many other autoimmune diseases, MS is associated with the presence of specific antigens of the major histocompatibility complex (Batchelor et al., 1978). In man the MHC region is the human lymphocyte antigen (HLA) gene cluster located on chromosome 6. The MHC proteins play a role in interactions between lymphocytes and in interactions between lymphocytes and antigen presenting cells. Three types of MHC molecules, class I, II and III, have been identified in both mice and man. Processed antigens in association with MHC class I products are primarily recognized by cytotoxic T-cells. Processed antigens associated with MHC class II products are recognized by T-helper cells. MHC class III genes are encoding for components of the complement system, a complex group of serum proteins which mediate a variety of inflammatory reactions.

In MS patients in Northern Europe, North America and Australia the disease is linked to MHC antigens Dw2, Drw2 and HLA-A3 and B7. The association of MS with HLA-A3 and B7 antigens has not been found in northern Italy and Israel

(Kurdi et al., 1977). Analysis of Drw antigens in northern Italy and Jordan has demonstrated a correlation between MS and MHC antigen Drw4. MHC antigen Drw6 is associated with MS in patients in Japan (Batchelor at al., 1978). The results indicate that the disease may be linked to more than one different genetic haplotype backgrounds, which means that there is not one single gene which increases the susceptibility to MS exclusively.

1.6.2.2 T-cell reactivity

T-cells can functionally be divided into cytotoxic T-cells (Tc) and T-helper (Th) cells. The Tc-cells are involved in the lysis of transformed or infected target cells. Th-cells function as regulatory cells and provide help to B-cells and T-cells to proliferate and differentiate. The T-cells recognize protein-antigens as processed peptides. The processing involves the uptake of antigens by antigen presenting cells, like B-cells, dendritic cells and macrophages, a subsequent controlled proteolytic fragmentation and the association of the immunogenic peptides with MHC proteins. The complex of immunogenic peptide and MHC will be expressed on the membrane of the antigen presenting cells. Subsequently, the immunogenic peptides in context of MHC proteins will be recognized by the T-cell receptor. MHC class I and II molecules itself do not have the ability to discriminate between self derived peptides and peptides originating from foreign antigens. As a consequence both self- and nonself peptides will be bound to the MHC molecules and presented to T-cell receptors.

In a normally functioning organism, no response will be evoked against self antigens, in other words the T-cell is tolerant to self-antigens. In general, two major mechanisms for induction of T-cell tolerance can be distinguished. A) In an early stage of development, the presentation of self antigens in the thymus to T-cells leads to the elimination of the T-cells which recognize the self-antigens. This process is called thymic tolerance or clonal deletion. However, not all self-antigens are transported to the thymus, or transported only in very low concentrations, especially self-antigens originating from immune privileged sites like the CNS or testis. B) For these antigens, another tolerance induction system, i.e. clonal anergy, is thought to be operational. T-cells, which are anergic to autoantigens, will not be activated when autoantigens are presented to them and as consequence autoimmunity will not occur. Although the precise mechanisms by which the T-cells are deleted or anergized remain to be revealed, it is possible that a defective T-cell tolerance induction or even a renewed sensitization of T-cells to myelin components may lead to the generation of an autoreactive T-cell population as observed in MS patients.

Autoreactive T-cells may contribute to autoimmunity in MS patients in two ways: directly as effector cell or indirectly as regulatory cell by activating autoreactive B-cells and other autoreactive T-cells. Extensive, partly conflicting, data have been presented concerning the presence, distribution and function of T-cells

specific for myelin components in peripheral blood and CSF, which seems to fluctuate with the clinical course of MS (Freedman and Antel, 1988). As compared to other putative antigenic components of the myelin sheaths, MBP is the most extensively investigated protein for its involvement in MS. MBP specific T-cells have been detected in the sera and cerebrospinal fluid of both MS patients and patients with other neurological diseases (Ota et al., 1990; Gorcy et al., 1983). Overlapping synthetic peptides of MBP were used to identify the antigenic determinants recognized by human T-cells. Some determinants (MBP aminoacid sequences 5-25, 35-47, 65-75 and 81-100) were recognized by T-cells derived from more than one individual (Burns et al., 1991). Because of the similarity in epitope specificity between T-cells isolated from various individuals, it was suggested that these epitopes are particularly immunogenic in humans. Zhang et al. (1990) used MBP fragments derived by proteolytic cleavage and synthetic peptides to reveal the epitope specificity of seventeen MBP reactive T-cell clones generated from four MS patients. The T-cell clones showed a striking similarity in reactivity with the C-terminal MBP peptide 149-171. Almost the same MBP epitope, sequence 154-172 was recognized by a majority of the MBP reactive T-cell clones derived from both MS patients and healthy subjects (Martin et al., 1990). The MBP sequence 149-171, which is recognized by human T-cells, overlaps the MBP epitope which can induce CNS demyelination in rhesus monkeys (Karkhanis et al., 1975). However, the association between the MBP epitope 149-171 and encephalitogenicity in humans remains to be established.

Myelin proteolipid protein (PLP) can induce a T-cell-mediated demyelinating disease in the Hartley guinea pig (Yoshimura et al., 1985), the Lewis rat (Yamamura et al., 1986) and in rabbits (van der Veen et al., 1986). Therefore, PLP is considered to be an important putative autoantigen in the pathogenesis of MS as well. T-cells reactive with PLP have been isolated from patients with early relapsing remitting MS (Trotter et al., 1991). PLP reactive T-cells were also detected in blood and CSF from control patients, but these cells were detected in much lower numbers as compared to the frequency in MS patients (Sun et al., 1991). No difference in reactivity to PLP peptide 139-151, a peptide which can induce demyelination in SJL/J mice, was measured between T-cells from peripheral blood from MS patients as compared to T-cells from normal subjects and from patients with other neurological diseases. These data indicate that the presence of T-cells reactive with putative CNS antigens in sera by itself is not leading to CNS demyelination. Possibly, the autoreactive T-cells are recruited specifically to the site of immune attack, i.e. the CNS tissues, in MS patients and not in healthy subjects or in patients with other neurological diseases.

1.6.2.3 B-cell reactivity

B-cells are characterized by their ability to produce and secrete antibodies. Circulating antibodies can be found in most body fluids, although the antibody concentration in the CSF of healthy subjects is relatively low. Antibodies are multifunctional molecules, consisting of two identical antigen binding sites (the Fab fragments) and an Fc portion which can activate complement and interact with Fc receptors, which are expressed on the surface of various cell types of the immune system. Neutrophils and most specialized antigen presenting cells express Fc receptors on their membranes, which allow them to phagocytose antigen-antibody complexes for intracellular degradation. Furthermore, antibodies can sensitize target cells for attack of cytolytic cells. The mechanism of this antibody mediated immune defence is called antibody dependent cellular cytotoxicity (ADCC). Several cells, like macrophages, eosinophils, cytotoxic T-cells, natural killer- and killer cells, can attack target cells making use of the ADCC mechanism.

Antibodies recognize self- or nonself antigens which are in solution or expressed on cell surfaces. The antigen binding to antibodies initiates a cascade of cellular reactions including G protein activation, phospholipase activation and Ca^{2+} mobilization, which leads to the ultimate activation of the B-cell. However, not all antigens can activate B-cells directly but require additional help of T-cells. These antigens are called thymus dependent (TD) antigens. The TD antigens will be internalized, processed and subsequently antigenic peptides are presented in context of MHC class II molecules to Th-cells. In turn, the recognition of the processed antigens in association with MHC class II molecules by the Th-cell receptor activates $CD4^+$ Th-cells. The activated Th-cells will produce and secrete cytokines and will express the gp39 membrane activation marker (Armitage et al., 1992, Noelle et al., 1992). The binding of the gp39 marker to its B-cell ligand CD40, is required for an optimal stimulation of B-cells to proliferate and to differentiate (Paulie et al., 1989) . Direct B-cell activation without help of other cells can be accomplished by mitogenic components like lipopolysacharide (LPS) or by crosslinking the membrane bound antibodies on the B-cell by antigens. These kind of antigens are called the thymus independent antigens. The putative autoantigens in MS are considered to be, TD antigens and as a consequence clonal deletion or anergy of Th-cells will prevent the ultimate activation of autoreactive B-cells as well.

Although the role of antibodies and B-cells specific for myelin components in MS is less well documented, there is circumstantial evidence of antibody and antibody forming cell (AFC) involvement in the process of demyelination. Persistent oligoclonal IgG synthesis in the CSF (Walsh and Tourtelotte, 1983) and a high antibody specificity index (ASI) (ASI is the ratio QAb:QIgG, whereas QAb and QIgG are the CSF/serum quotient of specific antibodies and total IgG levels respectively; Felgenhauer and Reiber, 1992) are indicative for a sustained B-cell activation within the CNS of MS patients. In both CSF and sera of MS patients,

antibodies directed against components of brain tissue (Henneberg et al., 1991) and a variety of putative CNS antigens like cerebellar soluble lectin (Zanetta et al., 1990a, 1990b), glycolipids (Endo et al., 1984; Hirsch and Parks, 1976), MBP (Link et al., 1990), PLP (Sun et al., 1991) and myelin associated glycoprotein (Baig et al., 1991) were detected. Warren and Catz detected antibodies in MS patients, directed against myelin components, particularly during exacerbations of clinical signs (Warren and Catz, 1986). In contrast, this correlation was not observed by Link and coworkers (Link et al., 1990). Gorcy reported the detection of elevated frequencies of anti-MBP antibodies in CSF of MS patients and patients with other neurological diseases (OND) as compared to healthy controls (Gorcy et al., 1983). Others were not able to demonstrate a statistical difference in anti-MBP antibody titers in CSF (Chou et al., 1983) nor, in the frequencies of anti-MBP AFCs in blood (Jingwu et al., 1991) between MS patients and healthy controls. Recently MBP specific antibodies were isolated from suspensions of CNS tissues of MS patients (Warren and Catz, 1993).

1.6.2.4 Molecular mimicry

The autoantigen itself is not always required for the activation of autoreactive cells. It is possible that autoreactive cells are activated by cross-reactive epitopes of pathogenic agents like bacteria and viruses. This phenomenon, sharing of antigenic determinants, is called molecular mimicry. Making use of computer analysis to compare microbial protein sequences to human protein sequences, many homologies have been identified. Subsequently, making use of peptides corresponding to these shared epitopes, immunological cross reactivity has been identified for some of those sequences. These findings indicate that epitopes of nonself antigens, which are homologous to self proteins, may drive autoreactive B- or T-cell clones to expand and cause autoimmune pathogenesis. Therefore, molecular mimicry is considered to be a potentially important mechanism which plays a role in the induction of MS.

When the mechanisms of elimination or inactivation of selfreactive cells are somehow overridden, the recognition of autoantigens, or cross reactive epitopes of foreign antigens, leads to the activation of selfreactive T- and B-cells. However, the presence of activated autoantigen reactive T- and B-cells or antibodies *per se* does not imply the organism will automatically develop an autoimmune disease. One of the reasons the autoimmune response will not occur, is the lack of an appropriate amount of selfantigen, or antigen with cross reactive epitopes, to stimulate the selfreactive cells continuously. Furthermore, the presence of autoantigen-reactive T- and B-cells and antibodies, does not necessarily imply that these cells or the autoantibodies are involved in the induction or enhancement of the autoimmune disease. Especially the role of autoantibodies in the process of several autoimmune diseases remains unclear. Myasthenia gravis is the only autoimmune disease, in which the direct involvement of anti-acetylcholine antibodies as pathogenic effector

molecules was established (Fuji et al., 1988). However, throughout the spectrum of autoimmune diseases studied, ranging from organ specific autoimmune diseases like Hashimoto's thyroiditis (Delves and Roitt, 1984) and multiple sclerosis to non-organ specific autoimmune diseases like systemic lupus erythematosus (SLE) (Lehman et al., 1984), autoantibodies have been detected. Although these findings make the production of autoantibodies one of the hallmarks of autoimmunity and MS in particular, it is not known whether these autoantibodies are an epi-phenomenon, a secondary event, or if they are directly involved in the pathogenesis of the disease. In addition, the fact that autoantibodies can easily be detected, not only in the serum of persons with MS but also in the serum of healthy individuals, makes the role of autoreactive antibodies and B-cells in the process of autoimmunity even more controversial.

1.6.3 Infectious agents

Although genetic components can not be excluded, the occurrence of MS epidemics also points to an infectious agent, as the environmental factor. MS epidemics were observed on the Faroe islands and Iceland (Kurtzke and Hyllested, 1979; Kurtzke et al., 1980b). On the Faroe islands no cases of MS were diagnosed before 1940, while three epidemics occurred in 1946, 1957 and 1969 respectively, following occupation by the British military during World War II. These data suggest that an infectious agent was introduced into the young population of the Faroe islands during the World War II. Although many viruses have been implicated in MS, including measles (Salmi et al., 1973; Miyamoto et al., 1976; Adams et al., 1970), herpes (Catalano, 1972), canine distemper virus (Cook et al., 1979), corona virus (Burks et al., 1979) and rubella (Horikawa et al., 1973), no particular virus can be regarded as the MS causing antigen. On the other hand, it has been hypothesized, that numerous neurotropic budding viruses could provide a carrier effect for CNS (host derived) glycolipid haptens. In turn, glycolipids presented to the immune system, whether or not in combination with viral membrane proteins, could induce an anti-glycolipid immune response, which subsequently leads to demyelination (Webb and Fazakerley, 1984). According to this hypothesis, many different viruses may be involved in the etiology of MS.

1.7 Animal models

Direct sampling of MS target tissues is almost impossible due to the inaccessibility of the CNS. Observations made in experiments performed with CSF and blood samples of MS patients give secondary information on processes in the CNS only. These problems have led to the extensive use of animal models to study the process of demyelination. Animal models provide important knowledge with respect to basic mechanisms used by the immune system to discriminate between

self and foreign antigens. Although there are many differences between the experimental animal diseases and their human autoimmune counterparts, the experimental animal models are generally the only tool to obtain knowledge concerning the fundamental mechanisms involved in disease induction or enhancement.

The principle of experimental autoimmune disease induction in general is based on interference with the immune system in such a way that the naturally occurring tolerance is overruled. Autoimmune diseases can be induced by two distinct procedures. First, by the adoptive transfer of autoreactive cells to naive recipients. Autoreactive cells can be isolated from animals with autoimmune diseases or can be isolated after *in vitro* stimulation with autoantigen. Second, autoimmune diseases can be induced by the artificial presentation of an excess of autoantigen to the immune system. The introduction of autoantigen can be accomplished in two ways. Autoantigens can be introduced indirect by the inoculation of viruses which cause tissue damage, upon which as a consequence autoantigens will be released and presented to the immune system. Subsequently, due to tissue damage, autoantigens will be released, presented to the immune system and autoantigen reactive cells will be activated. Inoculation of animals with Theiler's murine virus (Dal Canto and Lipton, 1975), Semliki Forest virus (Suckling et al., 1978) or murine coronavirus JHM (Weiner, 1973) induces experimental autoimmune diseases which resemble human autoimmune demyelination. However, the most commonly used method for the introduction of autoantigens is the direct administration of autoantigens to animals by immunization. Because a normal functioning animal will be tolerized to the administered autoantigens, the use of strong adjuvants is often an essential requirement.

Experimental autoimmune encephalomyelitis (EAE), which mimics the effector phase of MS, is generally considered to be the best available animal model for MS. The most commonly general used method for EAE induction is subcutaneous immunization of susceptible rodents or primates with whole CNS homogenates or with separated and purified autologous or heterologous CNS protein components like MBP and PLP, emulsified in Freunds complete adjuvant (Paterson et al., 1976). The precise mechanisms which lead to the damage of myelin sheaths remain to be investigated. However, it is hypothesized that the autoantigens in the inoculum will be presented to T-cells, which will proliferate and differentiate. The autoantigen primed T-cells, and probably antigen specific B-cells as well, will enter the CNS (Meyermann et al., 1987). A second contact of these immune competent T- and B-cells with CNS autoantigens, to which they were primed, will activate the T- and B-cells again and an immune response against the antigens will be the result. Another method for EAE induction is the adoptive transfer of myelin reactive T-cells isolated from animals immunized with myelin components or obtained after *in vitro* activation with myelin components (Mokhtarion et al., 1984; Veen et al., 1989; Pettinelli and McFarlin, 1979). More recently it became possible

to induce EAE in susceptible rodents also with selected sequences corresponding to amino acid sequences from myelin sheath proteins. Although the myelin sheath consist of several protein components, EAE induction has been established by the immunization with amino acid sequences of MBP and PLP only (table 1).

In this thesis the EAE model in SJL/J mice, induced by immunization with PLP synthetic peptides, has been used. The use of synthetic peptides for induction of autoimmune diseases has many advantages as compared to the use of intact autoantigens for induction of autoimmune diseases. Induction of autoimmune diseases with synthetic peptides in general leads to a higher disease incidence than use of the intact autoantigen. Since the autoantigen is well demarcated, the immune response is restricted and as a consequence less complicated to analyse.

Although it is possible to induce EAE with individual proteins and with peptide sequences, this does not imply that these proteins or sequences are the specific antigens or epitopes responsible for the EAE induction as observed in EAE elicited with spinal cord homogenate. To identify the 139-encephalitogenic proteins and epitopes in the spinal cord homogenate, the *in vitro* proliferative responses of lymphoid cells isolated from animals with spinal cord homogenate induced EAE were determined to myelin sheath proteins and MBP and PLP peptides (Whitham et al., 1991b). Lymph node cells only responded with PLP peptide 139-151, but not with PLP peptide sequence 141-152 nor with MBP or MBP synthetic peptides. Mononuclear cells, isolated from the CNS of animals with spinal cord induced EAE, reacted preferentially with PLP synthetic peptides. In addition, PLP peptide 151 specific cells from mice immunized with spinal cord homogenate transferred EAE into naive recipients. These data suggest that this PLP sequence is one of the major encephalitogenic epitopes in SJL/J (H-2^s) mice.

Based on the course of appearance of clinical signs, two types of EAE can be distinguished. First, acute EAE which is characterized by only one single period in which clinical signs are manifest. Second, chronic relapsing-remitting EAE (CR-EAE), which is characterized by two or more periods with clinical or neuropathological signs (Raine, 1985; Polman et al., 1988). The course of appearance of clinical signs in CR-EAE resembles the appearance of clinical signs in MS more closely as compared to the course of appearance of clinical signs in acute EAE (Alvord, 1985). The severity of clinical signs, the sequence of appearance of clinical signs and which clinical signs will be manifest is highly dependent on the antigen dose, the myelin components in the inoculum and the animal strain. In SJL/J mice, immunized with PLP peptide 139-151 in Freunds complete adjuvant, the first clinical signs are in general observed 11-12 days after immunization. Clinical signs include: partial or complete paralysis of the tail, paresis of the hind legs, partly or complete paralysis of the hind legs and paralysis up to the diaphragm. Clinical signs in SJL/J are always accompanied with a significant body weight loss. Although some animals die due to EAE, most animals usually recover 20-21 days after immunization.

Table 1
Encephalitogenic aminoacid sequences for EAE induction in rodents.

myelin basic protein sequences:

strain	haplotype	residues	reference
mouse			
PL/J	$H\text{-}2^u$	1-37	Fritz et al., 1985.
		1-11	Zamville et al., 1986.
SJL/J	$H\text{-}2^s$	84-102	Su et al., 1992.
		87-114	Kono et al., 1988.
		87-98	Kono et al., 1988.
		88-98	Kono et al., 1988.
		89-169	Pettinelli et al., 1982.
		89-104	Su et al., 1992.
		89-98	Kono et al., 1988.
		91-103	Su et al., 1992.
		91-104	Kono et al., 1988.
SWR	$H\text{-}2^q$	89-101	Jansson et al., 1991.
B10.T(6R)	$H\text{-}2^q$	89-169	Pettinelli et al., 1982.
NFR/N	$H\text{-}2^q$	89-101	Jansson et al., 1991.
B10RIII	$H\text{-}2^r$	89-169	Pettinelli et al., 1982.
		89-101	Jansson et al., 1991.
rat			
Lewis	RT-1l	1-88	Levine et al., 1990.
		45-88	Levine et al., 1990.
		63-88	Levine et al., 1990.
LOU/M	RT-1w	110-129	Hashim et al., 1991.
Buffalo		87-99	Jones et al., 1992.

proteolipid protein sequences:

strain	haplotype	residues	reference
mouse			
PL/J	$H\text{-}2^u$	35-47	Zamville et al., 1988.
		43-64	Whitham et al., 1991a.
SJL/J	$H\text{-}2^s$	139-151	Tuohy et al., 1988, 1989.
		141-150	Kuchroo et al., 1991.
		141-149	Kuchroo et al., 1991.
SWR	$H\text{-}2^q$	103-116	Sobel et al., 1991.

Not all strains of an animal species are equally susceptible to EAE induction. Dunkin Hartley guinea pigs, Lewis rat and SJL/J, SWR, NFR/N and PL/J mice are susceptible to induction of EAE (Raine and Stone, 1977; Offner et al., 1987; 1989; Brown and McFarlin, 1982; Cross et al., 1991; Fritz et al., 1985; Zamvill et al., 1986). Brown Norway rats and BALB/C mice are resistant to EAE induction. In general the differences in susceptibility to EAE induction are attributed to MHC class II restriction. On the other hand, quantitative changes in local MHC class II expression might influence the susceptibility to disease as well. Animals which are highly susceptible to EAE induction appears to have an increased expression of MHC class II molecules in the CNS (de Groot et al., 1991). Furthermore, susceptibility to EAE induction is decreased in aging mice. In addition, there are some indications that a decreased susceptibility to EAE induction is caused by a disturbed antigen recognition of T-cell receptors (Endoh et al., 1990; Zamvill and Steinmann, 1990).

1.8 Outline of the thesis

Many abnormalities affecting the immune system are associated with MS. These abnormalities can also be observed in other autoimmune diseases. In addition, immune competent cells directed against components of the CNS can be detected in MS patients. These observations suggest that MS is a CNS specific disease, in which autoimmune processes are involved. Furthermore the CNS of MS patients is characterized by perivascular lymphocytic infiltrates. Although these infiltrates contain both T-cells, B-cells and macrophages, immunological research has been focused largely on the involvement of T-cells in the pathology of MS only. This T-cell focused research is mainly based on experiments in which EAE was induced by passive transfer of myelin component specific T-cells to naive recipients. In addition, there are many similarities in immuno- and neuro-pathological changes, during the process of demyelination, in both EAE animals and MS patients. Though the presence of oligoclonal IgG in the CSF is still used as one of the characteristics to diagnose MS, not much is known about the specificity of these antibodies and the role of antibodies and antibody forming cells in the pathogenesis and etiology of MS. The potential effector functions of antibodies together with the detection of antibodies directed against several putative antigens in the circulation of MS patients suggests that antibodies may play a role in the pathogenesis of multiple sclerosis. However, in MS, antibodies can only be pathogenic if the antibodies or the AFCs can enter the CNS tissues. Therefore we decided to develop methods for the detection of antigen specific antibody forming cells in CNS tissue section of MS patients (chapter 2).

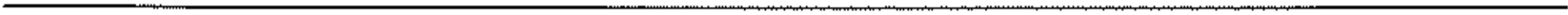
Antigen specific cells can be detected applying immunohistochemistry making use of antigen-enzyme conjugates. The approach and the principle of this technique are introduced in chapters 2.1 and 2.2. Improvement of the technique and the construction of MBP-enzyme conjugates are described in chapters 2.3 and 2.4, respectively. MBP-enzyme conjugates were used to localize MBP specific cells in

autopsy CNS tissues of MS patients (chapter 2.5). The significance of the localization of myelin component specific AFCs in the CNS of MS patients is discussed in chapter 2.6.

After confirmation of the presence of autoantigen specific AFCs in the CNS tissues of MS patients we investigated the presence of Th-cells, necessary for B-cell activation. Activated $CD4^+$ Th-cells express the gp39 marker on their membranes. Monoclonal antibodies directed against gp39 have been shown to block the ability of Th-cells to activate B-cells, indicating that the interactions between the Th-cell gp39 marker and its CD40 ligand on B-cells are important to T-cell dependent B-cell activation. Using enzyme labelled monoclonal antibodies directed against the gp39 marker, $gp39^+$ T-cells were detected, in the same affected CNS tissues of MS patients in which we have detected the MBP-specific AFCs (chapter 3).

Based on the assumption that MS is an autoimmune disease most therapeutic approaches are focused on intervention of the immune response. One of the approaches to modulate the immune response is the blockade of co-stimulatory signals required for lymphocyte activation. In chapter 3, we studied the blockade of the gp39/CD40 by anti-gp39 monoclonal antibodies on the development of EAE in mice.

An other approach of immune modulation is suppression of the immune response by induction of tolerance. Tolerance can be induced by oral administration of putative auto-antigens. Live transformed microorganisms can be used for an efficient oral delivery of antigens. The presentation of antigens delivered by microorganisms to the immune system may result not only in tolerization of the immune system to the presented antigens but may result in the induction of a cellular and/or humoral immune response to the presented antigens as well. The principles and advantages of using microorganisms as antigen carrier are described in chapter 4.1. A plasmid transformation system was developed for *Lactobacillus* species, to generate a safe non-pathogenic, live antigen delivery system. In addition, *Lactobacillus* was examined for the ability to deliver a model antigen, trinitrophenyl (TNP), via the oral route to the immune system to generate a humoral immune response (chapter 4.2). Trinitrophenyl specific AFCs were localized *in situ* after oral administration of *Lactobacillus*-TNP constructs (chapter 4.3). In chapter 4.4 we studied the induction of tolerance by the oral administration of genetically modified *Lactobacillus* secreting antigen. In addition, we have studied the influence of the oral administration of encephalitogenic PLP peptides and *Lactobacillus* on the development of EAE in mice. Finally, implications of the data described in chapters 2-4 is discussed in chapter 5 for our understanding of MS-pathogenesis and development of therapeutic approaches.



CHAPTER 2

IN SITU B-CELL RESPONSES IN MS AND EAE

CHAPTER 2.1

INTRODUCTION TO *IN SITU* B CELL RESPONSES IN MS AND EAE

B-cells in MS

Although the main interest in experimental autoimmune encephalomyelitis (EAE) and multiple sclerosis (MS) has been focused on T-cell immunity, many of the immunological abnormalities found in MS patients are B-cell related and these clinical features are routinely used for diagnostic purposes. Since the putative autoantigens in MS are considered to be TD antigens, autoreactive B-cells will only be activated in cooperation with T-cells and therefore B-cell reactivity may be regarded to be a read-out system for T-cell reactivity. On the other hand B-cells or their products may function, directly or indirectly, as effector cells in MS immunopathology as well. First B-cells may induce T-cell reactivity by functioning as autoantigen presenting cells. Secondly autoantigen reactive antibodies may be involved in complement activation and in antibody-dependent cell mediated cytotoxicity (ADCC). Many cells, including NK-cells, possess Fc receptors which allow them to act as effector cells in ADCC.

High levels of IgG in the cerebrospinal fluid (CSF) and a high anti-body specificity index are typical hallmarks of MS. High antibody levels have also been detected in central nervous system (CNS) tissues of MS patients. Warren and Catz (1986) detected antibodies in the CSF of MS patients, directed against myelin components, particularly during exacerbations. Such a correlation was not observed by Link and coworkers (Link et al., 1990). Górny et al (1983) reported the detection of antibodies directed against myelin basic protein (MBP) and oligodendrocytes in CSF of MS patients and patients with other neurological diseases. Others were not able to detect antibodies directed against MBP in CSF (Chou et al., 1983) or were not able to detect a difference between the frequencies of anti-MBP AFCs in blood (Jingwu et al., 1991) of MS patients and healthy controls. The IgG in the CSF of MS patients appears to be oligo-clonal when examined by electrophoresis. This can be explained in two ways. The observed oligoclonal response is due to an uncontrolled activation of a restricted number of B-cells clones, which are not necessarily reactive with auto-antigens. On the other hand, it may be possible that due to the release of a limited number of autoantigens, during the process of demyelination, only a limited number of B-cell clones are activated. Although the antigen specificity of most of the oligoclonal CSF IgGs remains to be identified, the specificity of at least a part of the antibodies in CSF, CNS tissues and serum has been revealed.

Antibody specificities

In both CSF and sera of MS patients, antibodies directed against components of brain tissue (Henneberg et al., 1991) and a variety of other putative CNS antigens were detected. Myelin basic protein (MBP) is probably the most intensively investigated antigen in this respect. Oligoclonal anti-MBP IgG was detected in the CSF of 32% of a panel of MS patients, but not in the CSF of a control group of patients with other neurological diseases (Cruz et al., 1987). Epitope specificity of anti-MBP antibodies in CSF of MS patients was revealed making use of synthetic peptides (Warren and Catz, 1992a). In MS patients, epitope specificity of non-antigen complexed anti-MBP antibodies from CSF was identical to the epitope specificity of non-antigen complexed anti-MBP antibodies isolated from cerebrum of MS patients. Both tissue derived complexed anti-MBP antibodies from cerebrum and antigen complexed anti-MBP antibodies from CSF are directed to a more restricted number of epitopes as compared to non-complexed anti-MBP antibodies in MS patients (Warren and Catz, 1993). Also immune complexes in sera of MS patients were found to contain MBP (Dasgupta et al., 1983). Complexed anti-MBP antibodies, were also found in sera of MS patients (Coyle and Procyk-Dougherthy, 1984). However, this finding was not confirmed in recent experiments (Geffard et al., 1993).

Also other myelin components may play a role in immunopathology of MS. Apart from MBP, the major component of the myelin sheath proteins, the drastic loss of myelin associated glycoprotein (MAG), a minor constituent of the CNS myelin sheaths (Quarles et al., 1973), in plaques in MS patient CNS suggests that MAG is a target protein in MS immunopathology (Johnson et al., 1986; Moller et al., 1987). Antibodies to MAG have been detected in the CSF of MS patients (Wajgt and Gorny, 1983; Möller et al., 1989). Anti-MAG antibody forming cells (AFCs) together with anti-MBP AFCs were detected in the CSF of the same MS patient by Baig et al. (1991). In addition there are indications that myelin oligodendrocyte glycoprotein (MOG) might function as an autoantigen. MOG is a quantitative minor component of the myelin sheath and is similar to the M2 protein that it is a target for antibody mediated demyelination in guinea pig (Lebar et al., 1986). The presence of anti-MOG antibodies in the CSF of MS patients was reported by Xiao et al. (1991).

Another candidate autoantigen is proteolipid protein (PLP). This claim is mainly based on animal experiments. Like MBP, PLP can induce experimental autoimmune encephalomyelitis in animals when inoculated together with Freunds adjuvant (Paterson, 1976). PLP reactive B-cells were detected in patients with multiple sclerosis (Sun et al., 1991). Furthermore, antibodies reactive with a variety of other minor CNS components, including cerebellar soluble lectin and glycolipids have been detected in both serum and CSF of MS patients (Hirsch and Parks, 1976; Endo et al., 1984; Zanetta et al., 1990a; 1990b).

Titers of antibodies directed against a variety of viruses, including mumps (Norrby, 1978), measles (Salmi et al., 1973) and herpes (Catalano, 1972) in CSF or MS patients are increased as compared to the antibody titers in CSF of healthy controls. However, it was not possible to associate the presence of anti-virus antibodies with one of the IgG bands and thus far it has not been possible to demonstrate that one particular virus is associated with the etiology of MS.

Detection of antibodies

Autoantigen specific antibodies or the autoantigen specific B-cells should be detectable in elevated quantities in the vicinity of affected areas of the CNS in patients with active disease and undetectable in those areas of the CNS which are not affected, if anti-autoantigen antibodies are involved in the pathogenesis of demyelination. However, most investigations into antibody and/or AFC involvement in the process of demyelination performed so far, were restricted to the determination of the presence or specificity of antibodies and AFCs isolated from sera, CSF or CNS tissues. However, from these studies no firm conclusion can be drawn with respect to possible correlations between the local presence of autoantigen specific antibodies or AFCs and the induction or enhancement of local tissue damage in the CNS. Therefore we decided to determine both localization and antigen specificity of AFCs in CNS tissue.

The classical methods of detecting antibody forming cells *in situ* are based on the use of monoclonal antibodies specific for the isotype of the antibodies produced by the AFCs, which allow visualization of the cells of interest. These assays are carried out on fixed tissues. The fixation procedure used should not change the antigenicity of the antibodies produced by the AFCs. The visualization may be obtained with direct or indirect procedures. The direct procedure uses the "AFC detecting" antibodies conjugated directly to an enzyme, fluorescent label or a radiolabel. The indirect procedure uses the labelled antibodies in the second step to detect the "AFC detecting antibodies".

A disadvantage of both direct and indirect methods is that these methods require purified antibody preparations for the conjugation procedure. Furthermore, the capacity of the antibodies to bind antigen may be lost or perturbed due to the conjugation procedure. This last disadvantage can be circumvented by using the unlabelled staining procedure as was described by Sternberger et al. (1970). In this procedure anti-enzyme antibody and enzyme is applied in an additional third step.

No matter which of the above remarked classical methods will be used for the *in situ* detection of AFCs, detection by using labelled antibodies will only provide quantitative information on the localization of the AFC and the isotype of the produced antibodies but not on the antigen specificity of the antibodies. By using a newly developed immune histochemical technique for the *in situ* detection of AFCs, it is possible to obtain information on the localization and antigen specificity of the

AFCs simultaneously.

The principle of this technique, which utilizes antigen-enzyme conjugates, is described in chapter 2.2. An improvement of the conjugation procedure, which was made applicable even for insoluble peptide antigens, is described in chapter 2.3. In chapter 2.4 we describe the construction and validation of MBP horse radish peroxidase (MBP-HRP) conjugates. Subsequently the MBP-HRP conjugates were used to localize MBP specific AFCs in autopsy brain tissue sections of MS patients (chapter 2.5). The relevance of the presence of autoantigen specific AFCs in the CNS of MS patients will be discussed in chapter 2.6.

CHAPTER 2.2

NEW IMMUNO-ENZYME-CYTOCHEMICAL STAININGS FOR THE *IN SITU* DETECTION OF EPITOPE SPECIFICITY AND ISOTYPE OF ANTIBODY FORMING B-CELLS IN EXPERIMENTAL AND NATURAL (AUTO)IMMUNE RESPONSES IN ANIMALS AND MAN

E Claassen, K Gerritse, JD Laman and WJA Boersma

TNO Medical Biological Laboratory, Rijswijk, The Netherlands

J Immunol Methods 150:207-216, 1992

Summary

Immuno-enzyme staining techniques in histo-cytochemistry can be used for the detection of antigen specific antibody forming cells (AFCs) in tissue sections. Here we describe the epitope specific detection of AFCs in various tissues based on the application of antigen-enzyme conjugates. Using antigen-enzyme conjugates rare AFCs can easily be detected and enumerated. Even multiple antibody specificities can be visualized in a single tissue sample and tissue compartments. This approach allows careful localization and investigation of compartimentation of AFC subpopulations. We established the development of a method for the detection of epitope specific AFCs in human lymphoid and central nervous system tissues in order to investigate how B-cells may contribute to the pathogenesis of autoimmunity in man.

Introduction

As we reviewed before, we have developed a direct double-immunoenzyme approach for the detection of antigen specific antibody forming B-cells (AFC) *in situ*. In animal studies with model antigens (such as albumins and γ-globulins) specificity of the antibodies in tissue sections could be demonstrated after incubation with antigen-enzyme conjugates and their isotype could be simultaneously determined by using an anti-immunoglobulin (Fc-chain specific)-enzyme conjugate followed by appropriate (double) enzyme-chemistry (Van Rooijen and Claassen, 1986).

By means of hapten-enzyme and hapten-carrier conjugates made with haptens such as trinitrophenyl (Claassen and Van Rooijen, 1984), penicilloyl (Boorsma et al.,

1986) and arsonate (Brown and Claassen, 1988), immune responses against thymus-dependent and T-independent (Claassen et al., 1986a-b) and/or soluble versus particulate carriers (Claassen et al., 1987a) could be studied. The method was adapted for use in other lymphoid tissue such as that associated with the gut (Jeurissen et al., 1985; Gerritse et al., 1991a) or the lung (Van der Brugge Gamelkoorn et al., 1986).

After modification in conjugation and staining procedures this technique was also used for the simultaneous *in situ* detection of crossreacting idiotype and anti-idiotype AFC. In other assays the rapid clearance of Id-anti-Id complexes and the technical demands of quantitating auto-anti-Id makes these studies very complicated (Brown and Claassen, 1991).

In rabbits we studied B-cell development (Claassen et al., 1986c) and persistence in adoptive immunity (Claassen et al., 1987b) and we were also able to demonstrate both an experimental and a spontaneous auto-anti-allotype immune response which could not be detected by means of any other assay (Claassen and Adler, 1988).

In all our studies special emphasis was placed on the development and localisation of AFC in lymphoid tissues as a function of the carriertype which was used (Van Rooijen et al., 1989; Laman et al., 1991b). The relation of these AFC with different other splenic celltypes was established *in vivo/in situ* by means of several double staining techniques as summarised in table 1. We found the *in situ* approach offered a number of advantages such as the unequivocal detection of rare AFC (1:10^7), detection of multiple (crossreactive- or auto-) antibody specificities in a single sample and the determination of tissue compartments, clone size and distances between cells (Brown and Claassen, 1988). However, the use of experimental antigens and animal studies, as described in the above studies, could not solve major questions arising in virus induced pathogenicity (Laman et al., 1989) or autoimmunity. We therefore started out to develop a method for the detection of epitope specific AFC *in situ* in human lymphoid tissues involved in natural (auto)-immuneresponses. In this paper we will outline the principle of a new technique for the detection of such AFC, based on the use of synthetic peptides, with special emphasis on (previously unpublished) detailed technical demands and properties.

Principle of the method for epitope specific detection of antibodies

As shown schematically in figure 1 the method works as follows: selected synthetic peptides (9-25 aminoacids long) are covalently coupled to enzymes such as horseradish peroxidase (PO), alkaline phosphatase (AP) or β-galactosidase (GL). To obtain a configuration that most resembles that of the native protein a coupling method which provides a random orientation of the peptides is preferred (Zegers et al., 1990). Cryostatsections (8 μm, -20^{0}C) of lymphoid tissue from experimental

animals (Laman et al., 1990; Gerritse et al., 1991) or human autopsy/biopsy material (Laman et al., 1991a) are incubated with the conjugate. When antibodies cross-reactive with the peptide-epitope(s) are present in the section the conjugate is bound (1b) and the cell will be coloured after appropriate cytochemistry. Antibodies visualised in this way can be both cytoplasmic (AFC) or extracellular, the latter in the form of e.g. immune-complexes (colour pictures in: Laman et al., 1990) or on memory B-cells (Liu et al., 1988). By making use of an isotype specific antibody (labeled with a different enzyme) directed against the Fc portion of the Ig molecule the isotype can be simultaneously determined (1c), due to the resulting intermediate violet colour in cells binding both conjugates (singly stained cells are specific for either the peptide or the isotype under study).

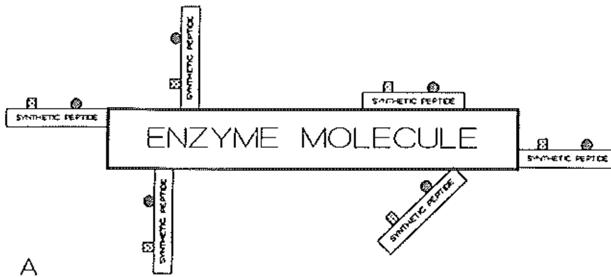
Selection of peptides

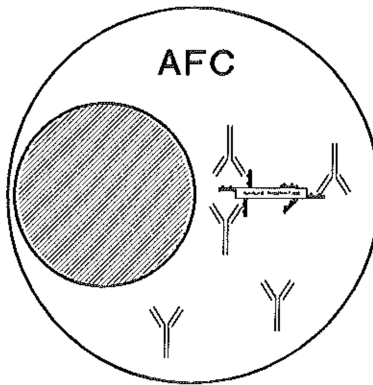
Peptides are selected primarily based on the biological question under study, e.g. neutralising epitopes from (putative, subunit-) vaccines. Numerous peptide sequences possibly involved in auto-immunity (e.g. from myelin basic protein) have already been put forward and tested in *in vitro* assays and/or animal experiments (Wucherpfennig et al., 1991). In the case of a suspected molecular mimicry involved in auto-immunity or pathogenesis (Laman et al., 1989) one should select those epitopes from the protein that resemble native antigenic structures, e.g. parts of a virus that have sequence homology with human proteins (Lernmark et al., 1988). An educated guess, based on comparison in databases, can in this case be supported by making use of methods for the *in vitro* study and simulation of antigen/protein processing and the release of immunogenic peptides by proteolytic enzymes (Van Noort et al., 1989).

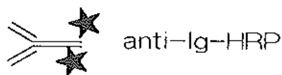
For the selection of the exact peptide-sequences and the construction of these conjugates we used computer prediction programs and knowledge obtained in a parallel line of research in which we generated diagnostic antibodies by means of selected synthetic peptides, as we reviewed in detail elsewhere (Boersma et al., 1991). A number of new coupling procedures was developed to amplify the proportion of antibodies that recognise the synthetic peptide and also crossreact with the native protein (Deen et al., 1990; Zegers et al., 1990). By adapting these procedures we were able to conjugate synthetic peptides to enzymes such as AP

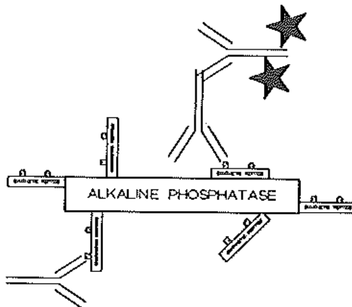
Figure 1 (next page)
Synthetic peptides are covalently coupled, in a random orientation, to enzymes such as horseradish peroxidase, alkaline phosphatase or β-galactosidase. Cryostatsections of lymphoid tissue are incubated with the conjugate, and this can be bound by antibodies cross-reactive with the epitopes present in the peptide. By making use of an isotype specific antibody (labeled with a different enzyme) directed against the Fc portion of the Ig molecule the isotype can be simultaneously determined. The number of B-cell epitopes, recognised by the antibodies in the section may vary from one to several and is depending on the length and nature of the peptide.

Figure 1









C

(Laman et al., 1990), HRP and GL (Laman et al., 1991c). Controlled conditions enabled the production of conjugates without loss of enzyme activity and with excellent binding capacities of the synthetic peptides to antibodies *in vitro* (ELISA) and *in situ*. These conjugates were successfully used to detect both AFC and immunecomplexes directed against selected, B-cell epitopes of HIV-1 proteins such as gp120 (Laman et al., 1991a).

Selection and coupling of enzymes

Basically we have employed three enzymes: alkaline phophatase, Horse radish peroxidase and β-galactosidase (table 1 and 2).

Alkaline phosphatase

As table 1 shows AP was most frequently used, mainly because it can be readily modified with the dialysis glutaraldehyde method to provide simple coupling through aminoresidues present in immunoglobulins (Claassen and Adler, 1988) and peptides (Zegers et al., 1990). The advantage of this modification of the classical one-step glutaraldehyde method are that no homopolymers (enzyme-enzyme or peptide-peptide) are formed and much less antigen is needed (Zegers et al., 1990). Furthermore, this conjugation can also be performed in 8M ureum with otherwise poorly soluble peptides without significant loss of enzyme activity (Gerritse et al., 1991b). In addition to this AP can be revealed as a bright transparent blue (Claassen et al., 1986b) or red (Brown and Claassen, 1988) colour, both extremely well suited for immuno-enzyme-cytochemical double and triple stainings (for colour pictures Laman et al., 1991b). The red reaction product obtained through the use of Fast Red TR is also fluorescent and can thus be used for quantitation or fluorescent double/triple labelings (Claassen, 1991).

Horseradish peroxidase

In double staining the blue reaction product of AP we have found HRP in combination with the red immunodecoration by 3-amino-9-ethylcarbazole to yield best results. The more frequently used dye diaminobenzidine is not suited for double staining with AP substrates in our method because the dye is not transparent but (electron)dense. Coupling of peptides to HRP can not be obtained through aminoacid residues on HRP (e.g. by glutaraldehyde) since not enough (only 1-2) are available. Coupling of synthetic peptides should be performed by making use of the periodate oxidation method (Laman et al., 1991c). HRP is more difficult to couple but on the other hand it is simple to use, cheap (also the substrate), can be easily amplified and the conjugates can be frozen down (-20^{0}C).

Table 1
SIMULTANEOUS DETECTION OF SPECIFIC AFC AND OTHER CELLTYPES BY IMMUNOENZYME DOUBLE STAINING

Cell type	Marker	Enzyme	Antigen	Enzyme	Colours	Species	Selected reference
AFC	isotype	(various possible combinations and species)					Van Rooijen and Claassen, 1986
	Ag specificity	(or Ab specificity for three non crossractive epitopes)					Claassen et al., 1986d
	idiotype	AP	arsonate-KLH	HRP	B, R, V	mice	Brown and Claassen, 1988
	allotype	HRP	TNP-KLH	AP	R, B, V	rabbits	Claassen et al., 1986c
	α-allotype	HRP	allotype	AP	R, B, V, -	rabbits	Claassen and Adler, 1988
	α-idiotype	HRP	idiotype	AP	R, B, -	mice	Brown and Claassen, 1991
crossreactive B-cells	antigen specificity	HRP	related antigen	AP	R, B, V	rabbits	Van Rooijen et al., 1989
auto-reactive B-cells	self-antigen/peptide	AP	viral protein	HRP	B, R, V	humans	Laman et al., 1991a
resting B-cells	membrane IgM	HRP	TNP-ficoll	AP	R, B, V	mice	Laman et al., 1991b
memory B-cells	α-antigen IgM	HRP	various	AP	R, B, V	rats	Liu et al., 1988
resting T-cells	Thy-1.2, CD4, CD8	HRP	TNP-ficoll/KLH	AP	R, B, V	mice	Van den Eertwegh et al., 1991b
CK producing T-cell	α-interferon-γ	AP	various	HRP	B, R, -	mice	Van den Eertwegh et al., 1991a-b
natural killer cells	asialo-GM-1	AP	TNP-ficoll	AP	R, B, V	mice	Van den Eertwegh et al., 1991a
all macrophages	acid phosphatase	-	TNP-liposomes	AP	R, B, -	mice	Claassen et al., 1987b
macrophages of MZ	MAb ED3	HRP	TNP-ficoll/KLH	AP	R, B, -	rats	Claassen et al., 1986b
antigen	α-antigen MAb	GL	TNP-ficoll	AP	G, B, -	mice	Laman et al., 1991b

Ab = antibody; Ag = antigen; AP = alkaline phosphatase; α = anti; CK = cytokine; HRP = horseradish peroxidase; GL = β-galactosidase; B = blue; R = red; G = green-blue; V = violet; - = no single cells double stained; KLH = keyhole limpet haemocyanin; MAb = monoclonal antibody; MZ = marginal zone of the spleen; TNP = trinitrophenyl.

Table 2
APPLICATIONS OF ANTIGEN-ENZYME CONJUGATES IN THE DETECTION OF ANTIGEN SPECIFIC ANTIBODIES.

Antigen	Analyte	Sensitivity[a]	Linearity	Precision	Interferences	Selected reference
IN SITU TECHNIQUE						
Hapten arsonate	HRP[b]	10	10-50	100%	Background over 200 μg/ml	Brown and Claassen, 1991
TNP	HRP[c]	50	50-200	100%	Occasional natural α-TNP Ab	Claassen and Van Rooijen, 1985
	AP	2	2-25	100%	Endogenous AP in gut and kidney	Claassen and Van Rooijen, 1984
Peptide	AP	8	8-40	100%	Adjuvant induced AFC (controls)	Laman et al., 1990
	HRP/GL	5/10	10-40	100%	Adjuvant induced AFC (controls)	Laman et al., 1991c
Peptide in 8M urea or DMF	AP	5	10-25	100%	Pro-zone effect at high concentrations	Gerritse et al., 1991b
Protein < 160 kd	AP/HRP	5	10-20	100%	-	Van Rooijen and Claassen, 1986
160 kd (IgG)	AP/HRP	1	5-20	100%	-	Claassen and Adler, 1988
> 160 kd	HRP	50	50-200	NA	-	Voorbij, 1989
Bacteriae	AP/HRP	>100	-	-	Conjugates to large	Vervelde et al., submitted
Virusses	HRP	100	100-200	NA	Proteolytic enzymes present in antigen	Claassen et al., submitted
ELISA	HRP/GL	0.01	0.05-2	100%		Laman et al., 1991c
(competitive)	AP	NA	0.2-10	100%		Gerritse et al., 1991b
SPOT-ELISA	AP	5	5-15	100%	Purity of catching antibody	Vos et al., 1990

Legend see also table 1, a sensitivity is defined as the smallest amount of conjugate which still stains 100% of the AFC expressed in micrograms of conjugate per ml., linearity is defined as the range which is used for efficient double staining. b conjugate prepared by bridging with bovine serum albumin, c conjugate prepared by bridging with poly-l-lysine; DMF = dimethyl formamide; Ig = immunoglobulin G; NA = not assessed.

β-galactosidase

This enzyme was chosen because its greenish blue substrate provides an opportunity for cytochemical triple and quadruple stainings in combination with AP (blue + red) and PO (red/brown, NB: GL can be double stained even with diaminobenzidine). Furthermore, after acetone fixation of lymphoid tissue absolutely no background due to endogenous enzyme activity is observed. This latter fact provides the possibility for prolonged staining times (2-4 hours) and we have found assays with GL conjugates to be far more sensitive than those using PO or AP (Van den Eertwegh et al., submitted).

Fixation

For superior results we have found it very effective to keep the freshly cut tissue sections (on glass slides) overnight at room temperature in an atmosphere with very high humidity (e.g. closed box with wet tissues). Sections are air dried for a few hours before fixing or stored, wrapped in aluminium foil, at -20^{0}C. In almost all studies we have routinely used acetone (pro analyse quality, dried) fixation for 10 minutes at room temperature. In those cases where HRP was used 0.02% hydrogen peroxide was added, just before fixation, to inhibit virtually all endogenous peroxidase activity. However, acetone does not effectively inactivate all infectious agents and fixations like 0.37% v/v formaldehyde in PBS or 0.5% paraformaldehyde(w/v) in PBS, for 10 min at room temperature are the methods of choice under biohazard conditions (Laman et al., 1991d).

Immunochemistry and amplification

Enzymes are revealed as described in detail elsewhere, AP as brilliant blue with Naphtol AS MX phosphate as the substrate and Fast Blue BB Base (in the presence of levamisole to inhibit endogenous AP; Claassen et al., 1986c), or as bright red (also fluorescent) with Fast Red TR Salt (Brown and Claassen, 1988; Claassen, 1991) as the indicator dye. PO as bright red with 3-amino-9-ethylcarbazole (Claassen et al., 1986) or brown with 3,3-diaminobenzidine-tetrahydrochloride (Van Rooijen et al., 1989). GL as greenish blue with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Bondi et al., 1982).

The presumed insensitivity of direct techniques does not, in our hands, apply to the detection of AFC with antigen-enzyme conjugates, however in these rare cases where low staining intensity is observed a number of amplification steps can be used. Efficient amplification can be performed by prolonged incubation in the substrate medium only with GL, with both PO and AP it is advisable to change to fresh substrate solution for a second staining after 10 (PO) and 25 (AP) minutes respectively. Amplification before (and even after the first staining period) is also possible by a second incubation step with enzyme labeled anti-enzyme antibodies or

with enzyme-anti-enzyme-antibody complexes (PAP or APAAP; Mason and Sammons, 1978). Furthermore, specialised conjugates with several enzyme molecules can be prepared, e.g. by the use of poly-l-lysine polymerised PO (Claassen and Van Rooijen, 1984) or polymerisation of AP with glutaraldehyde.

Controls

Normal controls should include: enzyme only, substrate only, and at least one irrelevant conjugate (i.e. same enzyme with different peptide). In animal studies animals injected with medium and/or adjuvant-only serve as negative controls, in human studies lymphoid tissue-sections of healthy (or unrelated disease) persons must be included on the same glass slide. Furthermore the staining must be inhibited when free (or conjugated) antigen is added to the incubation medium.

In using synthetic peptides we found it necessary to incorporate a positive control, this being an enzyme conjugate of the native protein. Upon double staining with a peptide-enzyme-X conjugate and a native-protein-enzyme-Y conjugate, all cells staining for peptide reactivity should also stain for the protein. This serves as a 100% control for the three dimensional configuration of the peptide in relation to the native protein (Laman et al., 1990).

The double staining with an anti-isotype-enzyme conjugate does not only provide detailed information on the isotype distribution of the specific AFC but also enables confirmation of the fact that B-cells are stained (positive control). Further experimental and technical controls (when necessary) and additional caveats can be deduced from the paper on limitations of the antigen-enzyme approach (Van Rooijen, 1987).

Simultaneous identification of other markers

The main strength in the method for detecting specific AFC lies in the fact that particularly those cells actively involved in a given *in vivo* immune response against a defined antigen (or even a single epitope thereof) can be identified. This is in sharp contrast to studies using only anti-Ig stainings in which all immunoglobulin forming cells (also "bystanders") are detected (these cells can also be observed, as a bonus, in the double label technique we use, as singly stained cells, see fig. 1). In addition to this, the development and localisation of the specific AFC can be studied in relation to e.g.: the localisation of the antigen, antigen presenting cells (e.g. macrophages), T-helper and T-suppressor cells, cytokine producing cells and several other celltypes involved in induction or regulation of the response (as summarised in table 1). In some cases these double stainings may result in an intermediate, violet, colour, indicating that both markers under investigation reside in the same cell. Furthermore this can be done *in situ*, offering an integral picture, complete with cell-cell interactions *in vivo*, of the immune response (see also Laman et al., 1991b).

Applications of the technique

Assays

Antigen-enzyme conjugates can be effectively used for the detection of specific antibodies *in situ*, as described here (table 2), but also with superior results in developing of anti-Ig coated, antibody catching, ELISA (enzyme linked sorbent assay; Vos et al., 1990; Laman et al., 1991c) and the even more sensitive variant: the competition-ELISA (Gerritse et al., 1991b). In a spot-ELISA, which is an improved modern version of the classical plaque forming cell assay, these conjugates dramatically enhanced the sensitivity of the assay (10-20 times; Vos et al., 1990).

Experiments

The method we describe here is the only way to analyse the *in situ* interactions which occur during an immune response (Laman et al., 1991b). This is of particular importance when one wants to study the relation of antibody formation with local histopathological effects or pathogenicity (relation with clinical stage; Laman et al., 1991a). In all cases where one can not efficiently evaluate an immune response due to lack of material (or only frozen tissue available) this method is very suitable, since only little material is needed and the method is sensitive enough to pick up rare AFC. In those cases where antibodies bind directly to the target antigen (e.g. autoimmunity) and are therefore not observed in the blood or liquor (CSF) this is also the method of choice. Furthermore we use it in tissues where lymphoid cells (and AFC) are scarce such as skin and parts of the lamina propria of the gut.

Discussion

The use of antigen-enzyme conjugates for the detection of specific AFC *in vivo* offers several important advantages (Brown and Claassen, 1988; Claassen and Adler, 1988). The method we describe here for the epitope specific detection of AFC in animals and man with synthetic peptides has additional advantages over other methods. The main advantage is the fact that one can now study the immune response against natural antigens *in situ/in vivo*, in its anatomical context, in relation with other celltypes, mediators, or the inducing antigen itself. Also notable is the fact that this can be done without suspending the tissue, thereby destroying cell-cell interactions and possible feed-back mechanisms. Furthermore this is one of the few methods with which one can study antibody formation in stored material, thereby opening up the possibility of retrospective studies on frozen tissue.

This new technique for the epitope specific detection of antibodies *in vivo* is now used in routine analysis of biopsy material from HIV-1 infected persons. Furthermore, with synthetic peptides specific for CNS proteins, it is applied to study the initiation of auto-antibodies *in situ* in CNS-tissue from Multiple sclerosis patients.

CHAPTER 2.3

CONJUGATE FORMATION IN UREA: COUPLING OF INSOLUBLE PEPTIDES TO ALKALINE PHOSPHATASE FOR ELISA AND *IN SITU* DETECTION OF ANTIBODY FORMING CELLS

K Gerritse, MJ Fasbender, WJA Boersma and E Claassen

TNO Medical Biological Laboratory, Rijswijk, The Netherlands

J Histochem Cytochem 39:987-992, 1991

Summary

We report here a new method to produce synthetic-peptide/alkaline phosphatase (AP) conjugates in the presence of urea. The method allows the use of peptides which are not soluble to a sufficient degree in aqueous buffers. The presence of 8M urea during the construction of the synthetic-peptide/AP conjugates does not influence enzyme activity nor the affinity of the anti-peptide antibodies for the conjugated peptide. We demonstrate that these synthetic-peptide/AP conjugates can be used for detection of specific anti-peptide antibody forming cells (AFC) *in vivo*. This method for constructing enzyme conjugates with insoluble proteins or peptides suggests not only new possibilities for detection of specific AFC *in vivo*, but also for applications in receptor-ligand studies, ELISA (enzyme linked immunosorbent assay) and spot-ELISA for detection of antibody secreting cells *in vitro*.

Introduction

Since the first use of antibody-enzyme conjugates in an ELISA, there has been considerable progress in the application of protein/protein- and protein/peptide conjugates in immunological studies (Boorsma, 1988). Several investigations have indicated the potential of synthetic-peptide conjugates as vaccines (Askelöf et al., 1988; Heckels et al., 1990). Conjugates of synthetic-peptides and carrier-proteins can also be used to raise mono-specific polyclonal and monoclonal antibodies which cross-react with the native protein from which the peptide sequence was derived (Boersma et al., 1988; 1989; Gerritse et al., 1990a). In addition protein- or peptide/enzyme conjugates can be utilized for detection of specific antibody secreting cells *in vitro* or *in vivo*. Vos et al. (1990) used human-insulin/AP conjugates to detect murine cells secreting anti-insulin antibodies of different IgG subclasses in a spot-ELISA assay. Several protein/protein or protein/hapten conjugates have been

developed for immunocytochemical detection of specific AFC (for review, Van Rooijen, 1989). In a recent paper (Laman et al., 1990) we presented a new method which provides a tool for studying the fine specificity of the humoral immune response against any antigen in situ, by the use of synthetic-peptide/enzyme conjugates for the *in vivo* detection of antigen-specific AFC. However, to construct or use these conjugates it is, for most of the mentioned applications, necessary that the proteins or protein conjugates be soluble. One of the artificial methods to solubilize proteins or peptides, which have only a very low solubility in aqueous buffers, is the use of urea. Klasen et al. (1982, 1983) used urea to solubilize protein fragments and peptides for ELISA coating purposes to detect haemoglobin mutation specific antibodies.

In this report we describe a conjugation method for the construction of peptide/enzyme conjugates with the use of 8M urea in order to achieve a sufficient concentration of peptides for successful conjugation. Conjugation in the presence of urea was compared to conjugate formation in dimethylformamide (DMF) and phosphate buffered saline (PBS).

Material and methods

Peptide synthesis, purification, characterization and conjugation

The synthesis of peptides 082 and 013 (SP082, SP013) was carried out, according to the Biosearch synthesis protocol (Biosearch; San Rafael, California, USA) on polystyrene resin (1% crosslinking) to which the first amino-acid already was attached. SP082 is a 20-mer derived from human apolipoprotein E3-Leiden (Havekes et al., 1986) with the sequence Arg-Gly-Glu-Val-Gln-Ala-Met-Leu-Gly-Glu-Val-Gln-Ala-Met-Leu-Gly-Gln-Ser-Thr-Cys. SP013 is a 11-mer sequence, analog to amino-acid residues 231-241 of the heavy chain constant region 2 of human IgG2 with the sequence Ala-Pro-Pro-Val-Ala-Gly-Gly-Pro-Ser-Val-Cys (Boersma et al., 1989). Both sequences were synthesized with an additional cysteine at the carboxyterminal to enable coupling via the thiol residue. The synthesis was carried out with tertiary-butyl-oxycarbonyl amino-acids, following the solid phase method as described by Merrifield (1963). To prevent side chain formation during synthesis some of the amino-acids were protected as follows: arginine, tosyl (p-toluenesulfonyl); cysteine, p-methylbenzyl; glutamic acid, benzyl ester; serine and threonine, benzyl.

Peptide cleavage and side chain deprotection of the completed peptide was performed by hydrogen fluoride (HF) treatment as described by Bhatnager et al. (1983). After HF cleavage and purification, as we described before (Van Denderen et al., 1989), the amino acid composition of the synthetic peptide was determined by high performance liquid chromatography (HPLC) analysis according to the method of Janssen et al. (1986).

Coupling of SP082 to keyhole limpet haemocyanin (KLH) with the use of m-

maleimido-benzoyl-N-hydroxysuccinimide-ester (MBS) was carried out as described by Kitagawa and and Aikawa (1976) with some modifications (Van Denderen et al., 1989). The peptide-KLH conjugate (KLH-MBS-SP082) was used for immunization purposes. Coupling of peptide SP082 to bovine sera albumin (BSA) with the use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was performed following the method described by Deen et al. (1990). The peptide-BSA conjugate (BSA-EDC-SP082) was used for coating purposes in the inhibition ELISA.

Immunization

Female BALB/c mice aged 12-15 weeks, were kept in macrolon cages under 11 h dark/13 h light regimen at 20°C and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, The Netherlands) ad libitum. Experiments were performed under the auspices of the Dutch Veterinary Inspection, according to the law on Animal Experiments. In order to obtain tissue sections with anti-SP082 AFC's and specific anti-SP082 antisera, a group of four female BALB/c mice was immunized intraperitoneally with 75 μg KLH-MBS-SP082 conjugate emulsified in 50% specol (Bokhout et al., 1981) and boosted with the same amount of conjugate emulsion four weeks after the initial injection. Seven day's after the booster injection, mice were sacrificed by CO_2 euthanasia. Blood samples of the mice were collected by heart puncture and left for 30 min at 37°C. Sera were obtained after centrifugation at 300 g for 10 min at 4°C. The spleens were removed and stored in liquid nitrogen. Tissue sections of the organs were used to screen the different SP082-AP conjugates for staining properties.

Synthetic peptide - alkaline phosphatase conjugation

The peptide/AP conjugation was carried out as was described in detail by Zegers et al. (1990). Briefly, AP (10 mg.ml^{-1}) was dialysed against 0.2% glutaraldehyde in PBS for 16 h at 4°C. The activated AP was dialysed against PBS for several hours, and transferred to a reaction vessel. Peptide SP082 was added to urea (8M, pH 7.0, 1 mg.ml^{-1}), DMF (pH 7.0, 1 mg.ml^{-1}) and 0.1 M PBS (pH 7.2, 1 mg.ml^{-1}) respectively. The solutions/suspensions were stirred extensively and centrifuged (300 g) for 10 min at room temperature. The solubilized peptides were added to GA activated AP in a molar ratio of 100 molar equivalents. The mixtures were stirred for 16 h at 4°C. To block the remaining active GA groups, 100 μl 0.2 M lysine-HCl was added to the mixtures followed by a 2 h incubation period at 4°C. The excess peptide and lysine molecules were removed by dialysis against PBS. The obtained conjugates were encoded SP082-AP(urea), SP082-AP(PBS) and SP082-AP(DMF), dependent on the solvent in which the coupling was carried out. The peptide-enzyme conjugate formation was examined in an inhibition ELISA.

Alkaline phosphatase activity

The enzyme activity of the non-conjugated AP and of the peptide-AP

conjugates in the presence of urea was revealed on PVC microtiter plates (Titertek). An AP solution (14 $mg.ml^{-1}$ 3.0 M NaCl, Sigma P6774) and a solution of peptide-AP conjugate (5 mg $AP.ml^{-1}$ in PBS) were diluted (2 log dilutions from $1{:}1.10^4$) in 8M, 4M, 2M, 1M urea in PBS, respectively. The dilutions were added to a microtiter plate (50 μl/well) together with 50 μl para-nitro-phenylphosphate (Boehringer, Mannheim, FRG) (1 $mg.ml^{-1}$) in 10 mM Diethanolamine/1 mM $MgCl_2$, pH 9.8. After 30 minutes at 25°C the absorption was determined at 405 nm in a Titertek Multiskan apparatus (Flow Laboratories, Irvine, Scotland).

Inhibition ELISA

PVC microtiter plates (Titertek) were coated with BSA-EDC-SP082 (5 $\mu g.ml^{-1}$ in PBS, 50 μl/well). After overnight incubation at 4°C the supernatant was removed. To block non-specific binding a second coating of gelatine (5 $mg.ml^{-1}$ in PBS, 50 μl/well) was applied and incubated for 30 min at 25°C. The plates were washed five times with 0.05 % Tween-20 in PBS (PBS-T). Peptide-induced antisera diluted 1/100 in a solution of gelatin (0.1 $mg.ml^{-1}$ in PBS-T (PBS-T-G)) were preincubated for one hour at 25°C with dilutions of peptide/enzyme conjugates (32 to 0.03 $\mu g.ml^{-1}$ in PBS-T-G) and control antigens before they were added to the wells in 50 μl aliquots. The plates were incubated for 1 h at 25°C and washed five times with PBS-T. The plates were incubated for 1 h at 25°C with AP-labelled goat anti-mouse IgG (H + L) (KPL, Inc., Gaithersburg, USA) 1:2000 in PBS-T-G. After washing with PBS-T, 50 μl of a para-nitro-phenylphosphate (Boehringer, Mannheim, FRG) solution (1 $mg.ml^{-1}$ in 10 mM Diethanolamine/1 mM $MgCl_2$, pH 9.8) was added to the wells. After 30 minutes at 25°C the absorption was determined at 405 nm in a Titertek Multiskan apparatus (Flow Laboratories, Irvine, Scotland).

Tissue preparation and immunocytochemical staining

Cryostat tissue sections (8-μm) were fixed for 10 min in acetone containing 0.02 % (v/v) hydrogen peroxide. For determination of SP082 specific AFC in the spleen, tissue sections were incubated overnight at 4°C with various concentrations (100 to 12.5 $\mu g.ml^{-1}$) SP082-AP conjugates in a solution of BSA (1 $mg.ml^{-1}$) in PBS. After incubation the sections were rinsed thrice in PBS and incubated for 5 min at room temperature in PBS. Slides were stained for AP activity according to the method of Burstone (1958) with some modifications (Claassen et al., 1986d). A solution of 8 mg naphthol AS-MX phosphate in 400 μl N,N-dimethylformamide was added to 65 ml Tris-HCl buffer (0.1 M, pH 8.5, 37°C). A solution of 16 mg sodium nitrite in 400 μl H_2O was added to 16 mg Fast Blue BB base in 400 μl 2N HCl. After 1-2 min this mixture was added to the Tris-HCL buffer containing naphthol AS-MX phosphate. To prevent endogenous alkaline phosphatase activity, levamisole in a final concentration of 2 mM was added to the substrate solution. The solution was paper filtered to remove any precipitate. The slides were incubated vertically in Coplin jars for 20 min in substrate buffer at 37°C. To prevent over- or understaining,

staining was monitored with a light microscope. Staining was stopped by incubation in PBS for 3 min at room temperature. The sections were counterstained for 5 seconds with a solution of hematoxilin (Gurr). The slides were mounted in glycerol-gelatin. Staining with the three different peptide-AP conjugates were performed on alternating tissue sections of spleens of four mice. From each mice fifty sections per conjugate were stained. Control stainings were performed with the SP082-AP conjugates on spleen tissue sections from non-immunized mice and mice immunized with a non-relevant peptide conjugate. In addition spleen tissue sections from mice immunized with KLH-EDC-SP082 were stained with a non-relevant peptide-AP conjugate. AFC were counted and calculated per square mm using a digital image processor (Imaging Research Inc., Brock University, Ontario, Canada).

Results

Determination of alkaline phosphatase activity

The enzyme activity of non-conjugated AP in the presence of urea is depicted in figure 1a. The activity of AP dissolved in 4M, 2M and 1M is comparable with the activity of AP dissolved in PBS. However, when AP was dissolved in 8M urea the enzyme activity decreased. Enzyme activity of peptide-AP conjugate in the presence of urea is depicted in figure 1B. The peptide-AP conjugate dissolved in PBS, shows the lowest activity compared to the conjugates dissolved in urea. The activities of the conjugates are comparable from dilution $1:2.10^4$. Only in the $1:10^4$ dilutions are some differences observed. The highest activity was measured in the solution of peptide-AP conjugate dissolved in 8M urea.

Inhibition ELISA

The SP082-AP(PBS) and SP082-AP(urea) conjugates were able to inhibit the interaction of the anti-sp082 antisera with the coating of BSA-EDC-SP082 as was demonstrated in figure 2. The interaction was completely inhibited when an amount of 32 μg peptide-AP conjugate was used. The same degree of inhibition was observed with the (positive) control antigens BSA-EDC-082 and non-conjugated SP082. The 082-AP(DMF) conjugate inhibits the interaction of the anti-SP082 antisera with the coating of BSA-EDC-082 only if a large amount of conjugate is used in the preincubation period. An amount of 16 μg or less does not inhibit the interaction at all.

Immunocytochemical detection of SP082-specific AFC's

Specific anti-SP082 AFC were detected in all spleen tissue sections of KLH-MBS-SP082 i.p. immunized mice, independent of the type of SP082-AP conjugate used (figure 3 a-c). No AFC were detected in tissue sections of the same mice when stainings were performed with a non-relevant peptide/enzyme conjugate nor with tissue sections of non-immunized mice or mice immunized with a non-relevant

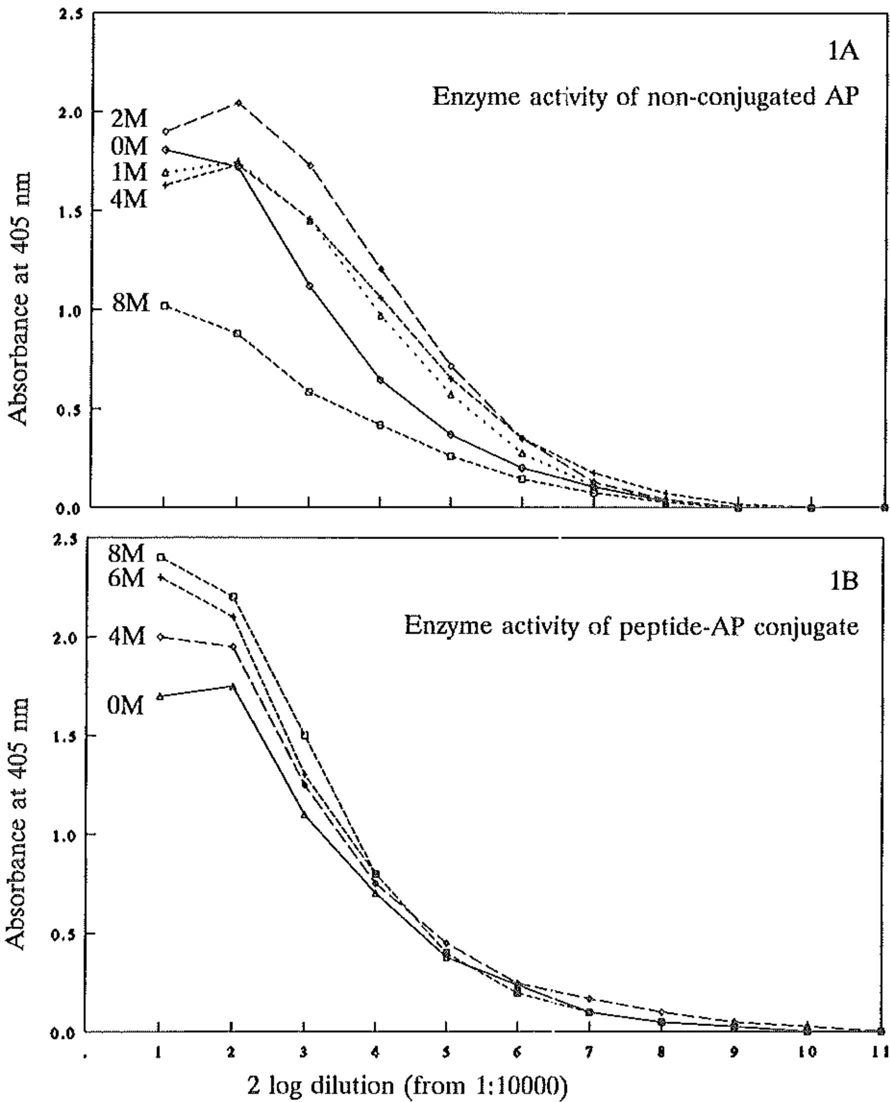


Figure 1
Enzyme activity of dilutions of the non-conjugated AP (figure 1A) and of peptide-AP conjugate (figure 1B) was determined in various concentrations urea (as indicated).

peptide conjugate stained with the SP082-AP conjugates. The localization of the AFC was similar to the localization as was described before (Van Rooijen et al., 1986). However, the number of specific AFC in the alternating spleen sections is dependent on the type and concentration of the conjugate used (figure 4).

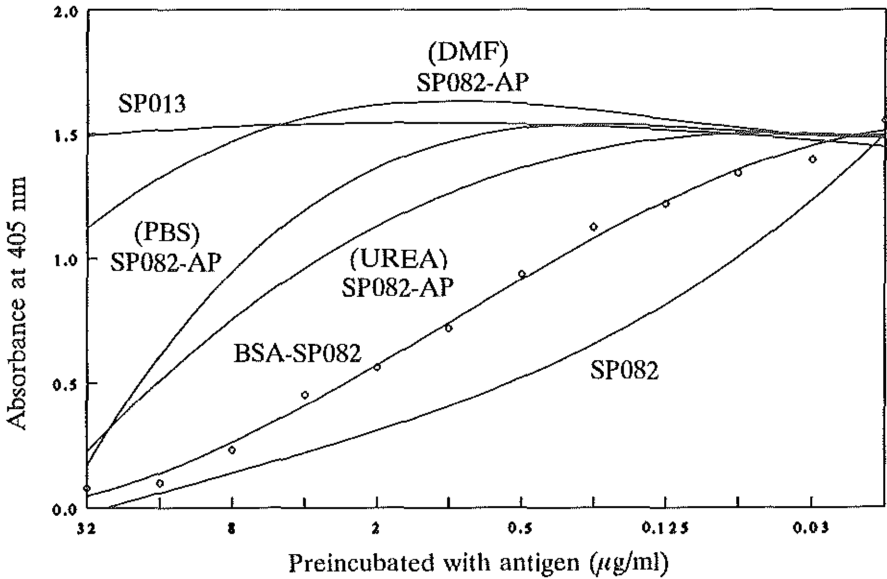
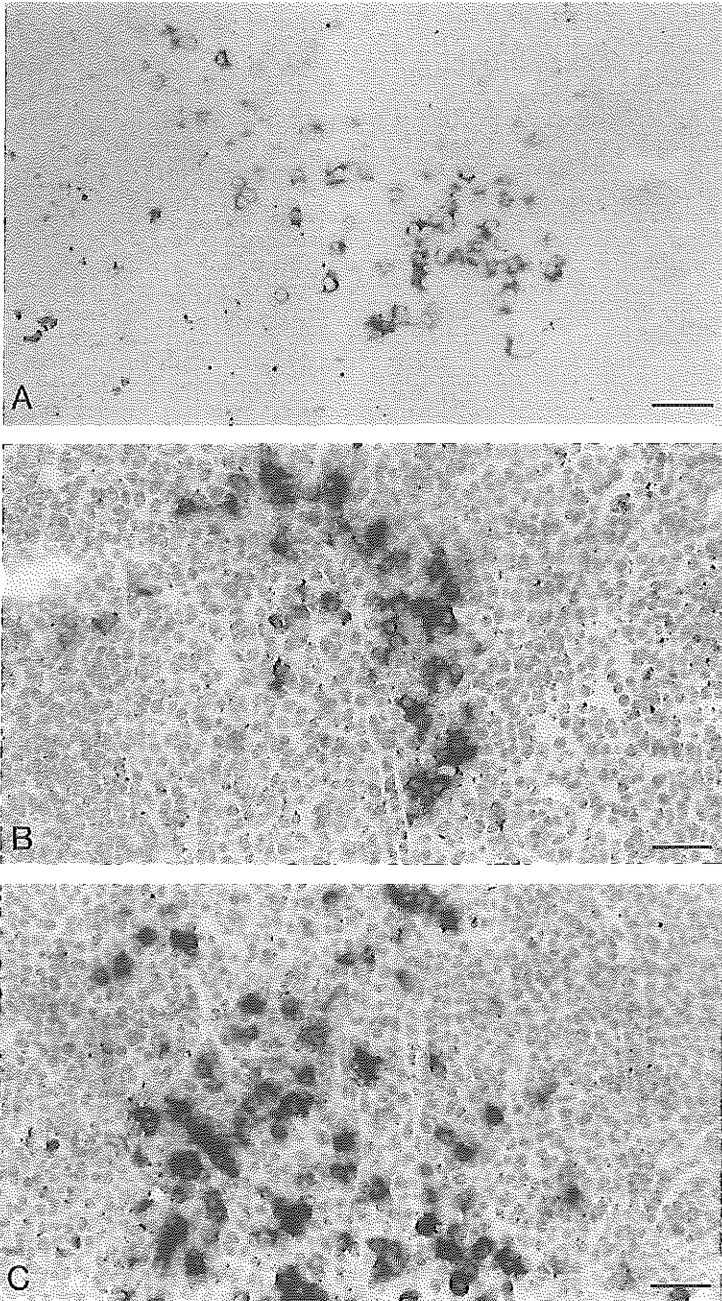


Figure 2
Coupling efficiency between SP082 and AP was evaluated by the capacity of the conjugates 082-AP(PBS), SP082-AP(DMF) and SP082(urea) to inhibit the interaction between the sp082 antisera and a coating of BSA-EDC-SP082. The BSA-EDC-SP082 and SP082 were selected as positive control inhibitors, SP013 was used as a negative control inhibitor. Alkaline phosphatase labelled goat anti-mouse IgG and para-nitro-phenylphosphate as substrate were used for detection.

Samples (concentration groups) were analysed by the two-sample Student's t-test for comparison of two empirical means in a normally distributed population (Sachs, 1984). Differences were assumed significant for $p < 0.05$. The highest number AFC were detected in the sections stained with the conjugates in a concentration of 12.5 $\mu g.ml^{-1}$, although the number AFC in these sections did not differ significantly from the number AFC detected in the sections stained with conjugates in a concentration of 25 $\mu g.ml^{-1}$. The number AFC detected with the SP082(urea) and SP082-AP(PBS) conjugates in a concentration of 25 $\mu g.ml^{-1}$ differ significantly ($p < 0.4$ and $p < 0.2$) from the number AFC detected with less diluted (50 $\mu g.ml^{-1}$) SP082-AP(urea) and SP082-AP(PBS) conjugates. Further dilution of the conjugates (6.25 $\mu g.ml^{-1}$ (not shown)) did not result in a significant increase in number of detected AFC.

Figure 3 (next page)
The spleens of mice, which were immunized ip with KLH-MBS-SP082 conjugate were removed on day seven after booster injection. Alternating tissue sections of spleens were stained with 082-AP(DMF) (a), 082-AP(PBS) (b) and 082-AP(urea) (c) respectively (conjugate concentration 12.5 $\mu g.ml^{-1}$). Original magnification x 100. Bar is 25 μm.

Figure 3



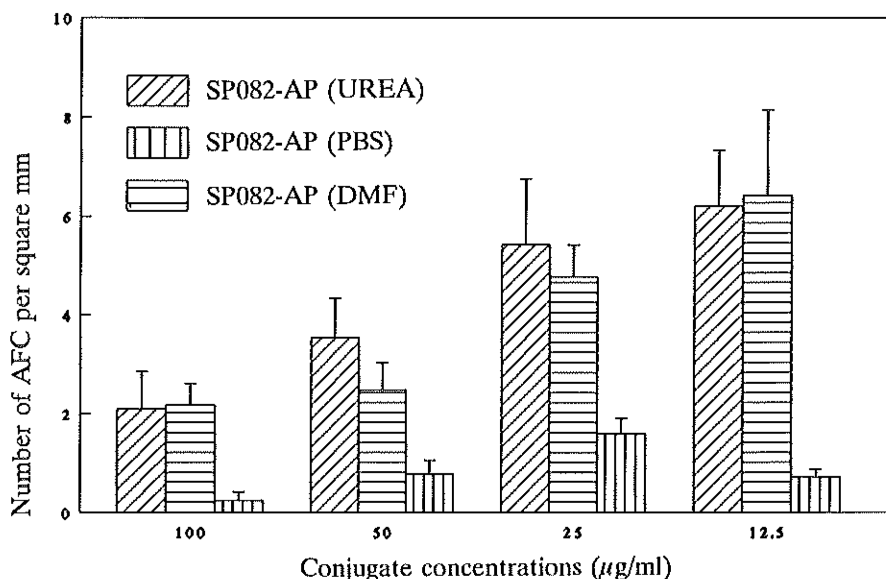


Figure 4
Number of specific anti-SP082 antibody forming cells in alternating spleen tissue sections, on day seven after booster injection, per square mm. The cells were detected by staining with dilutions of SP082-AP(urea), SP082-AP(PBS) and SP082-AP(DMF) conjugates. Standard error of mean is indicated.

In all conjugate concentration groups (group 100 to 12.5 $\mu g.ml^{-1}$) the lowest number of AFC was detected in the sections stained with the SP082-AP(DMF) conjugate. Although there are some small differences between the numbers AFC in the tissue sections stained with SP082-AP(urea) and SP082-AP(PBS) within one conjugate concentration group, those differences were not significant.

Discussion and conclusions

In this study we show that peptide/AP conjugate formation can be performed in the presence of 8M urea. The peptides were dissolved in urea to obtain a sufficient concentration necessary for a successful conjugation. Besides a suitable peptide concentration, two other basic conditions for an appropriate conjugation between an enzyme and a protein or peptide are required.

First, conjugation or the circumstances in which the conjugate formation occurs must not affect the biological-activity of the conjugated enzyme. As was revealed, the activity of the non-conjugated AP in the presence of 8M urea decreased remarkably compared to the activities of the enzyme measured in solutions with lower urea molarities or when measured in PBS. The activities of the enzyme

determined in 1M, 2M, 4M or in PBS are of the same magnitude (figure 1a). More important than the activity of the non-conjugated enzyme is the activity of the conjugated enzyme determined under conjugative conditions. We observed that the activity of the peptide/AP conjugate under these conditions was remarkably higher than the activity measured in PBS (figure 1b). This suggested better availability of the enzyme active sites in the conjugate in the presence of urea.

Second, the immunological properties of the conjugated protein or peptide must not be altered by the conjugation method. In other words, anti-peptide antibodies must recognize the conjugated peptide in the same way as the non-conjugated peptide. In the inhibition ELISA SP082-AP(urea)- and SP082-AP(DMF) conjugates, inhibited the interaction between anti-SP082 antibodies and a coating of BSA-EDC-SP082 in a way comparable to inhibition of the same reaction by non-conjugated SP082 (figure 2). This suggests that in spite of the presence of lysine blocked glutaraldehyde groups on the peptide and a possible sterical interference of the enzyme, the conjugation method did not influence the immunological properties of the conjugated peptide. In addition, the fact that both SP082-AP(urea) and SP082-AP(PBS) conjugates produced a comparable degree of inhibition, suggests that the same amount of peptide was conjugated to the enzyme in both conjugates. On the other hand, the SP082-AP(DMF) conjugate inhibited the interaction between the anti-SP082 antibodies and the coated antigen only when it was added in a high concentration, which indicates a lower peptide/AP ratio as compared to the other conjugates. Since the SP082-AP conjugates may in some instances block only one combining site of an anti-SP082 antibody and thereby allowing the other free site to bind to the BSA-EDC-SP082 coating of the microtiter plate, the AP from the SP082-AP conjugates may also contribute to the observed signal. The AP-peptide conjugate bound in this way would increase the apparent level of the signal in the assay. Although, such interference may be relatively small, it will only contribute in a positive way to the inhibition level. Therefore the conjugates might even be better than concluded. The use of a peroxidase labelled Goat anti-mouse conjugate in the inhibition assay instead of an AP labelled conjugate would rule out this presumed interference.

The presumed low peptide/AP ratio- or partial denaturation of the SP082-AP(DMF) conjugate was confirmed by staining of spleen tissue sections of BSA-EDC-SP082 immunized mice. The lowest number of specific AFC per square mm were detected by staining with SP082-AP(DMF) conjugate, independent of the conjugate concentration (figure 4). Furthermore there was a remarkable qualitative difference between the conjugates. The cells stained by SP082-AP(DMF) were not of the same intensity as the cells stained by SP082-AP(urea) and SP082-AP(PBS), respectively (figure 3a versus 3b-3c).

The SP082-AP(urea)- and SP082-AP(DMF) conjugate demonstrated a pro-zone effect when used in a concentration of 100 $\mu g.ml^{-1}$ up to 50 $\mu g.ml^{-1}$ and further dilution of the conjugates did not lead to a significant increase in the number of

detected AFC. A pro-zone effect (i.e. lower signal at higher concentration) is a frequently observed phenomenon in ELISA or cell staining experiments when antibodies or conjugates are used in high concentrations. There is no significant difference in the numbers of AFC per square mm detected by both the SP082-AP(urea)- or SP082-AP(DMF) conjugates independent of the conjugate concentrations. The presence of urea did not affect the enzyme activity nor the immunological properties of the coupled peptide and therefore this method can be used for the detection of AFC in situ (Van Rooijen and Claassen, 1986; Van Rooijen et al., 1989) and direct immunological double staining methods (Boorsma, 1988).

This method permits and expands the use of peptides and proteins, which are normally not readily soluble in aqueous solutions, for construction of peptide/protein and protein/protein conjugates for use in e.g. immunochemistry and sub-unit vaccine development. This study suggests that conjugation in the presence of urea for the formation of conjugates with proteins or peptides, may be a useful additional tool to the existing methods.

CHAPTER 2.4

IN SITU DETECTION OF SELF REACTIVE EPITOPE SPECIFIC ANTIBODY FORMING CELLS IN THE CENTRAL NERVOUS SYSTEM OF EAE RHESUS MONKEY

K Gerritse, B Slierendregt*, C Deen, MJ Fasbender,
WJA Boersma and E Claassen

TNO Prevention and Health, Leiden, The Netherlands
**TNO Institute for Applied Radiobiology and Immunology,*
Rijswijk, The Netherlands

EOS J Immunol Immunopharmacol 13:63-65, 1993

Summary

The structural protein components of the myelin sheaths, surrounding the neuronal axons, are considered to be putative target antigens for immune competent cells, which play a role in the pathogenesis of multiple sclerosis or experimental allergic encephalomyelitis (EAE). Whether or not antibody forming cells (AFCs) play a role in the pathogenesis of MS or EAE is unclear, because the experiments performed sofar concerned the detection of AFCs and antibodies, directed against myelin protein components, in the sera and/or cerebrospinal fluid only. By making use of myelin protein component-enzyme conjugates it is possible to detect anti-myelin protein component AFCs locally in the tissues of the central nervous system (CNS), which are actually affected. We have constructed and validated a conjugate of horse radish peroxidase and myelin basic protein (MBP), one of the major CNS myelin protein components, to determine the presence of the anti-MBP AFCs in the spleens of mice and cerebrum of rhesus monkeys after induction of EAE.

Introduction

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS), characterized by the occurrence of demyelinated areas (plaques), dissemination and perivascular accumulation of inflammatory cells. Although the exact etiology and pathogenesis of MS is unknown, it is generally accepted that autoimmunity is involved in the disease. The immune response in the cerebrospinal fluid is reflected by elevated numbers of mononucleated cells in 30-50 % of the patients and oligoclonal bands in almost 100 % of the MS patients.

One of the major components of CNS myelin to be considered a target

antigen is myelin basic protein (MBP). MBP has been a subject of many investigations because of its capacity to induce experimental allergic encephalomyelitis (EAE) in a variety of laboratory animals (Eyler et al., 1971). Whether anti-MBP antibodies play any role in the pathogenesis of MS or EAE is unclear. Results considering the detection of anti-myelin antibodies or anti-myelin antibody forming cells (AFC) are conflicting. However, most experiments concerned the detection of antibodies and cells in the peripheral blood and/or cerebrospinal fluid and not in the tissues in which the damage actually takes place. By making use of antigen enzyme conjugates it is possible to detect specific AFC's and extracelluar antibodies *in situ* (rev. Claassen et al., 1992). Here we describe the construction of MBP-horseradish peroxidase (PO) conjugates and the validation of these conjugates in immune-and CNS tissues of EAE-mice and rhesus monkey respectively.

Material and methods

Immunizations

Female SJL/J mice, 12 week old, were immunized with MBP to obtain anti-MBP AFC containing lymphoid tissues to examine antigen-enzyme conjugation. Mice were immunized in the peritoneal cavity (i.p.) three times with a two week interval with 200 μg bovine MBP (Sigma). The first dose was suspended in Freund's complete adjuvant (CFA, 9/11 v/v) with a total volume of 200 μl. The second and third dose were suspended in Freunds incomplete adjuvant. All mice received 1 h after each immunization a single dose of 2.10^9 killed *Bordetella pertussis* (i.p., 100 μl in PBS). Control mice received adjuvant suspended in PBS only. Mice were sacrificed on day 5 after the third immunization by CO_2 euthanasia, the spleen was removed, snap frozen in liquid nitrogen and stored at -70°C for further use.

Rhesus monkeys were born and raised in the Primate Centre TNO in Rijswijk, The Netherlands. Monkeys were immunized subcutaneous with an emulsion containing 1 ml antigen preparation and 1 ml CFA with 1 mg *Mycobacterium butyricum* (Van Lambalgen and Jonker, 1987). The inoculum was equally divided over four sites on the back. The antigen preparation consisted of 5 mg bovine MBP. Control monkeys received CFA suspended in saline only. Animals were sacrificed when they were moribund. Brain tissue samples were snap frozen and stored at -20°C for further use.

Conjugate formation

MBP was conjugated to PO with the two step glutaraldehyde (GA) method as was described by Van Rooijen and Claassen (1986) with some modifications. Briefly, PO was dissolved (10 mg) in 0.2 ml, 1.25% (v/v) GA in 0.1 M phosphate buffer (pH 6.8) and incubated overnight at room temperature. The PO-GA fraction was isolated on a PD-10 sephadex G-25M column (Pharmacia LKB, Uppsala, Sweden) after elution with PBS. The PO-GA fraction was concentrated by membrane filtration

(Amicon PM 30, Grace & Co., USA) to a final concentration of 10 $mg.ml^{-1}$. MBP (2 mg, Sigma, St. Louis, USA) was dissolved in a mixture of 40 μg 1M carbonate-bicarbonatebuffer (pH 9.8) and 360 μl PBS (concentration 5 $mg.ml^{-1}$). The MBP solution was added to 440 μl PO solution, 440 μl PBS and 160 μl 1M carbonate-bicarbonate buffer (pH 9.8) and stirred overnight at 4°C. A solution of 0.2M glycine (80 μl) was added to block the remaining active GA groups and incubated 2 h at room temperature. The MBP-PO solution was concentrated by membrane filtration (Amicon PM 30, Grace & Co., USA) to 5 $mg.ml^{-1}$, diluted (1:1 v/v) with glycerol and stored at -20°C.

MBP-PO coupling was examined by ELISA. PVC microtiter plates (Flow Laboratories, Irvine, Scotland) were coated with anti-MBP monoclonal antibodies (Boehringer Mannheim, Germany) (5 $\mu g.ml^{-1}$ PBS, 50 μl/well) and incubated overnight at 4°C. The supernatant was removed and the wells were incubated 30 min at 25°C with a solution of gelatine 0.2 $mg.ml^{-1}$ in PBS to block non-specific binding. The plates were washed (5x) with 0.01% Tween-20 (v/v) in PBS (PBST) and subsequently incubated for 1 h at 25°C with MBP-PO conjugate in a solution of gelatine (0.1 $mg.ml^{-1}$) in PBS. The plates were washed (5x) with PBST and a solution of o-phenylenediamine (2 $mg.ml^{-1}$, Eastman Kodak 107 8054, Rochester, NY, USA) in phosphate-citrate buffer pH 5.0 (37°C) was added to each well (50 μl/well). Prior to use 0.5 μl hydrogen peroxide (30 %) per ml substrate buffer was added to the solution. The plates were incubated in the dark at 25°C. After 15 minutes the absorption was determined at 450 nm in a Titertek Multiskan apparatus (Flow Laboratories, Irvine, Scotland).

Control incubations were performed with substrate solution only and an irrelevant protein-PO conjugate (proteolipid protein-PO, PLP-PO). In addition the MBP-PO conjugate was incubated on plates coated with an irrelevant catching antibody (anti-mouse Lambda chain).

Immune-histochemistry

Stainings were performed to detect specific anti-MBP AFC's. Eight micrometer cryostat mice spleen cross sections and monkey cerebrum coronal sections were fixed for 10 min in acetone containing 0.02 % (v/v) hydrogen peroxidase and dried for 10 min at room temperature. Fixed tissue sections were first pre-stained for non-specific peroxidase activity by incubation for 10 min in a solution of 4-chloro-1-naphthol, according to the method of Nakane, 1968. The reaction was stopped by incubation for 10 min in PBS. Subsequently tissue sections were incubated overnight at 4°C with MBP-PO conjugate dilutions in 0.1 % bovine sera albumin (BSA) in PBS (w/v). The slides were rinsed twice for 5 min in PBS and the PO activity was revealed with 3-amino-9-aminocarbozole, according to the method of Claassen et al. (1986d). The reaction was stopped after 10 min by incubation in PBS for 10 min at room temperature. All slides were counterstained with haematoxylin according to standard procedures.

In control experiments mice spleen tissue sections and monkey cerebrum tissue section were separately incubated with: substrate solution and an irrelevant protein-PO conjugate. Subsequently the specific peroxidase activity was inhibited by simultaneous incubation with non-conjugated MBP. In addition spleen and brain tissue sections of non-immunized mice and monkey respectively and spleen sections of mice immunized with an irrelevant antigen were incubated with the MBP-PO conjugate.

Results and Discussion

By making use of the two-step glutaraldehyde coupling method it is possible to construct MBP-PO conjugates. Since the conjugate could be detected in a direct ELISA (figure 1) in which the MBP part of the conjugate was recognized by an anti-MBP catching antibody, it could be concluded that the antigenicity of the MBP was not modified by the conjugation procedure.

To determine whether or not the conjugate could be used for *in situ* detection of specific anti-MBP AFC's, spleen tissue sections of EAE mice and brain tissue sections of EAE rhesus monkey were incubated with the conjugate. As is shown on photograph 1 and 2, groups of specific anti-MBP AFC's were detected in both tissues.

To prevent non-specific peroxidase staining the tissue sections were pre-incubated with 4-chloro-1-naphthol. The use of this substrate demonstrated the best visible difference between the specific and non-specific stained cells as compared to the use of other substrates (results not shown). The MBP-PO conjugates will now be used to detect specific anti-MBP AFC's in autopsy CNS tissues of MS patients. To reveal the epitope specificity of the specific MBP AFC's we have started experiments with conjugates of synthetic peptides derived from MBP and PO.

Photograph 1-2 (next page)

1) Spleen tissue sections of MBP immunized SJL/J mice were stained for non-specific peroxidase activity with 4-chloro-1-naphthol and specific anti-MBP AFC's respectively. Groups of anti-MBP AFC's are indicated with arrows.

2) Anti-MBP AFC's (red) were detected in brain tissue sections of EAE rhesus monkey. Specific cells were detected in areas of inflammation.

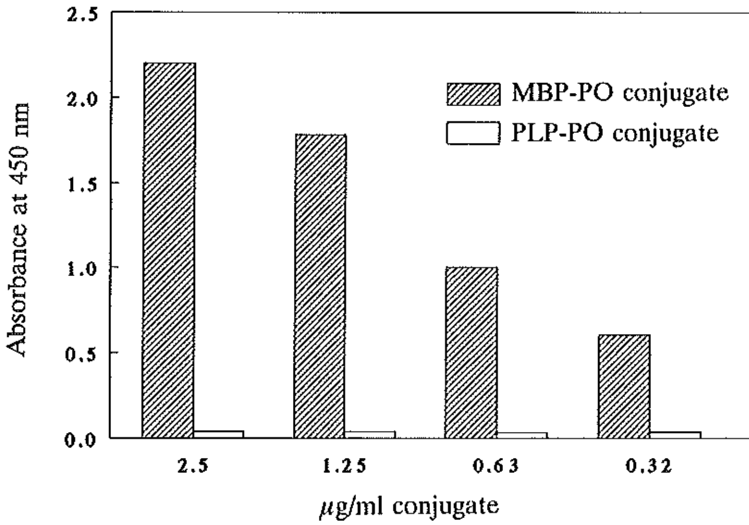
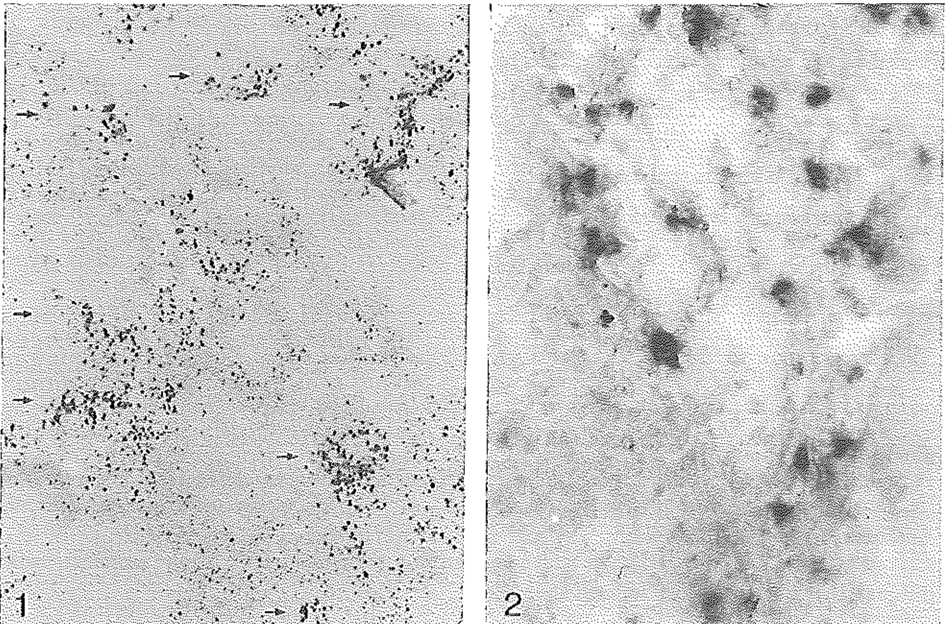


Figure 1
MBP-PO conjugate formation was analyzed in a direct ELISA on a coating of anti-MBP monoclonal antibodies. The MBP-PO conjugate was added to the wells in various concentrations as indicated on the horizontal axis. No signal was observed when a non-specific conjugate (PLP-PO) was added to the wells.

Photograph 1-2



CHAPTER 2.5

THE INVOLVEMENT OF SPECIFIC ANTI MYELIN BASIC PROTEIN ANTIBODY FORMING CELLS IN MULTIPLE SCLEROSIS IMMUNOPATHOLOGY

K Gerritse, C Deen, MJ Fasbender, R Ravid*,
WJA Boersma and E Claassen

TNO Medical Biological Laboratory TNO, Rijswijk, The Netherlands
**The Netherlands Brain Bank, Amsterdam, The Netherlands*

J Neuroimmunol 49:153-159, 1994

Summary

Irrespective of the large body of literature on the putative role of antibodies in the development of multiple sclerosis (MS), the detection of specific antibody forming B-cells (AFCs) in the central nervous system (CNS) tissues has not been described. In this study we show that autoantigen specific AFCs can be found in CNS tissue sections of MS patients. Applying a newly developed myelin basic protein (MBP)-enzyme conjugate technique, we have detected MBP specific AFCs in autopsy periventricular white matter and cerebellum tissue sections, of MS patients. We demonstrated the presence of MBP specific AFCs in CNS tissue sections in five out of twelve MS patients. Whereas no MBP specific AFCs were detected in CNS tissue sections of eleven patients with other neurological diseases, such as Parkinson's and Alzheimer's disease, nor in brain tissue sections of eight deceased persons without neurological diseases. In MS patients, anti-MBP AFCs were present in brain tissue sections both with and without plaques. The proportion of MBP specific AFCs in some of the MS patient brain tissues reached over 50 percent of all AFCs. The high relative frequency of the anti-MBP AFCs and their localization in periventricular white matter and cerebellum of MS patients only, suggests that anti-MBP AFCs represent a cell population, which could play an important role in MS immunopathology.

Introduction

Multiple sclerosis is an inflammatory disease which affects the central nervous system. The disease is characterized by breakdown of the multi-lamellar myelin sheaths around the axons, resulting in demyelinated lesions or plaques. The exact ethiology of the disease and pathogenetic mechanisms of demyelination are at

present unknown. It is widely accepted that immune- and autoimmune processes are involved in the development of the disease. The myelin breakdown is accompanied by an inflammatory response that includes T-cell subsets, B-cells and macrophages, resulting in perivascular infiltrates in the white matter parenchyma (Weller, 1985). Conflicting, data have been presented concerning the presence, distribution and function of T-cells specific for myelin components in peripheral blood and cerebrospinal fluid (CSF) which seems to fluctuate with the clinical course of MS (Freedman and Antel, 1988).

Although the role of antibodies and B-cells specific for myelin components in MS is less well documented, there is circumstantial evidence of antibody and antibody forming cell involvement in the process of demyelination. Persistent oligoclonal IgG synthesis in the CSF (Walsh and Tourtelotte, 1983) and a high antibody specificity index (Felgenhauer and Reiber, 1992) are indicative for a sustained B-cell activation within the CNS of MS patients. In both CSF and sera of MS patients, antibodies directed against components of brain tissue (Henneberg et al., 1991) and a variety of putative CNS antigens like cerebellar soluble lectin (Zanetta et al., 1990a; 1990b), glycolipids (Endo et al., 1984; Hirsch and Parks, 1976), myelin basic protein (MBP) (Link et al., 1990), proteolipid protein (Sun et al., 1991) and myelin associated glycoprotein (Baig et al., 1991) were detected. Of all putative CNS antigens MBP is the probably the most investigated antigen. Epitope specificity of anti-MBP antibodies in CSF of MS patients was revealed making use of synthetic peptides (Warren and Catz, 1992a). Epitope specificity of free anti-MBP from MS CSF is identical to the epitope specificity of free anti-MBP isolated from MS cerebrum. Both tissue bound anti-MBP antibodies from MS cerebrum and bound anti-MBP antibodies from MS CSF are directed to a more restricted number of epitopes as compared to non-bound anti-MBP antibodies (Warren and Catz, 1993). Immune-complexes in sera of MS patienten were found to contain MBP (Coyle and Procyk-Dougherthy, 1984; Dasgupta et al., 1983). Bound anti-MBP antibodies, i.e. as component of immune complexes, were also found in sera of MS patients (Coyle and Procyk-Dougherthy, 1984). However, the presence of MBP in immune complexes isolated from sera of MS patients could not be confirmed in recently performed experiments (Geffard et al., 1993).

Warren and Catz (1986) detected antibodies in MS patients, directed against myelin components, particularly during exacerbations. Such a correlation was not observed by Link and coworkers (Link et al., 1990). Gorcy reported the detection of elevated frequencies of anti-MBP antibodies in CSF of MS patients and patients with other neurological diseases (OND) as compared to healthy controls (Gorcy et al., 1983). Others were not able to demonstrate a statistical difference in anti-MBP antibody titers in CSF (Chou et al., 1983) nor in the frequencies of anti-MBP AFCs in blood (Jingwu et al.,1991) of MS patients and healthy controls.

Most investigations into antibody and/or AFC involvement in the process of demyelination performed, were restricted to the determination of the presence or

specificity of antibodies and AFCs in sera or CSF. From these studies no definite conclusion can be drawn with respect to possible correlations between the local presence of specific antibodies or AFCs and the induction or enhancement of local tissue damage in the CNS. We decided to determine the presence and specificity of AFCs in CNS tissue. To this end we made use of a newly developed *in situ* immunohistochemical technique (reviewed by Claassen et al., 1992).

Material and methods

Immuno-histochemistry

A myelin basic protein horse radish peroxidase (MBP-PO) conjugate was constructed (Gerritse et al., 1993) and used for the detection of specific MBP AFCs in a panel of CNS tissue sections from MS patients, control OND patients and non-neurological controls. 8 μm cryostat coronal sections of cerebrum periventricular white matter and cerebellum were fixed for 10 min in acetone containing 0.02 % (v/v) hydrogen peroxide and dried for 10 min at room temperature. Subsequently tissue sections were incubated overnight at 4°C with MBP-PO conjugate (20 $\mu g.ml^{-1}$) in 0.1 % bovine sera albumin in PBS (w/v). In order to determine the total number of IgG producing cells, serial sections were incubated with PO conjugated mouse anti-human IgG, clone 335-2.1 (2.5 $mg.ml^{-1}$ in 0.1 % bovine sera albumin in PBS (w/v)). All slides were rinsed three times for 5 min in PBS and the PO activity was revealed with 3-amino-9-aminocarbazole, as was described by Claassen (Claassen et al., 1986b). The reaction was stopped after 10 min by incubation in PBS for 10 min at room temperature. All slides were counterstained with haematoxylin according to standard procedures. Tissue sections were evaluated by light microscopy. The frequency of anti-MBP specific AFCs was calculated as a proportion of all IgG AFCs in serial sections.

In control experiments human brain sections were incubated with substrate solution only or with irrelevant protein (bovine serum albumin)-PO conjugate. Furthermore spleen tissue sections of MBP immunized mice, non-immunized mice and mice immunized with an irrelevant antigen (ovalbumin) were separately incubated with: the MBP-PO conjugate, substrate solution only and an irrelevant protein (bovine serum albumin)-PO conjugate. Subsequently, the specific binding of the MBP-PO conjugate was inhibited by simultaneous incubation with non-conjugated MBP.

Area determination

To express the number of AFCs per surface unit, the area of the brain tissue sections was determined by video imaging, using the micro computer imaging device and accompanying BRS2 software from Imaging Research Inc. (Brock University, St. Catherines, Ontario, Canada). The level of inflammation in the tissues was evaluated by counting the number of peri-venous inflammatory cell clusters. A cell cluster was

defined as an individual local accumulation of 10 cells or more. Cell and cluster numbers were used to calculate Spearman's Rho correlation coefficient.

Human tissues

Autopsy brain tissues were obtained from The Netherlands Brain Bank in Amsterdam (coordinator Dr. R. Ravid). Autopsy data are listed in table 1.

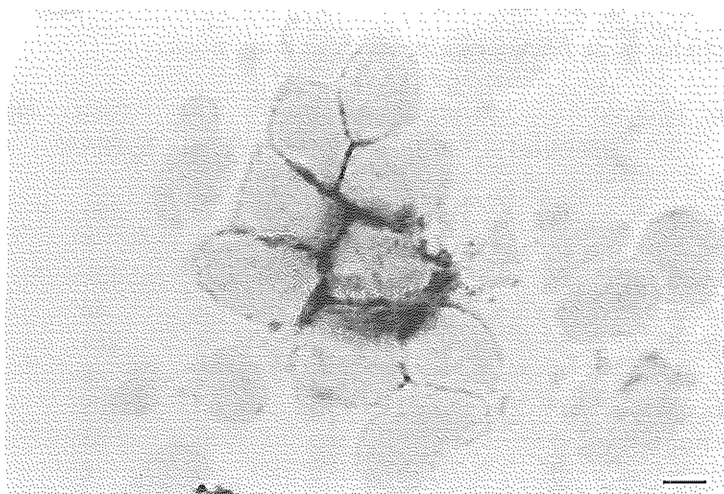
Results

Application of immuno-histochemistry with the MBP-PO conjugate, revealed MBP specific AFCs (photograph 1) in CNS tissue sections in five out of twelve MS patients. MBP specific AFCs were located in white matter only. The average area of cerebellum and cerebrum periventricular white matter tissue sections analyzed quantitatively per patient was 19 cm^2 and 43 cm^2 respectively. The frequency of MBP specific AFCs detected, ranged from 6 to 95 cells per 10 cm^2 (table 2). Although not consistent in all tissues tested, the majority of the MBP specific AFCs was detected in cell clusters, a minor part of the MBP specific AFCs was detected as individual cells and only few cells were present in perivascular cuffs. A significant positive correlation ($p < 0.05$) was determined for the frequency of MBP specific AFCs and the number of cell clusters (Spearman's Rho correlation coefficient = 0.58). Making use of the same immunohistochemical technique, anti-MBP AFCs could not be detected in a panel of twelve OND patients nor in a panel of eight non-neurological subjects (table 2).

The relative frequency of MBP specific AFCs ranges from 3 % to 52 %. In two MS patients (patient code: 8 and 9) the relative frequency of MBP specific AFCs was higher in the plaque containing tissues as compared to the non plaque containing tissues. In contrast, the relative frequency of MBP specific AFCs in plaque containing periventricular white matter tissue, of MS patient no.10, was lower as compared to the relative frequency of MBP specific AFCs in non-plaque containing periventricular white matter tissue (table 3). IgG producing cells, not specific for MBP, were detected, albeit in very small numbers, in the tissues of patients with other neurological diseases and non-neurological controls. However, no MBP specific AFCs were present in serial sections of these tissues, consequently the relative frequencies of MBP specific AFCs of the control groups are zero (table 3).

Patient code	Date of autopsy	Age[a]	First symptoms[a]	PMD[b]	Cause of death	Diagnose
MS patients						
1	122687	65	37	39.00	Cardiorespiratory failure	Multiple sclerosis
2	022090	57	24	24.00	Food inhalation	Multiple sclerosis
3	nk	62	nk	14.50	Pneumonia	Multiple sclerosis
4	123187	87	52	12.00	Bronchopneumonia	Multiple sclerosis
5	091288	45	25	11.30	nk[c]	Multiple sclerosis
6	120991	62	nk	10.10	nk	Multiple sclerosis
7	112190	55	nk	9.10	nk	Multiple sclerosis
8	030192	59	38	7.30	Septic shock	Multiple sclerosis
9	081791	63	nk	5.50	Bronchopneumonia	Multiple sclerosis
10	062791	57	21	5.20	nk	Multiple sclerosis
11	052288	43	23	5.00	Hepatic carcinoma	Multiple sclerosis
12	091290	41	34	4.45	Dehydration	Multiple sclerosis
mean:		58	32	10.05		
Patients with other neurological diseases						
13	062190	89	--	4.55	Vascular problems	Alzheimer disease
14	052990	78	--	4.10	Sepsis/Decubitus/Cachexia	Parkinson disease
15	071290	79	--	4.00	Pneumonia	Alzheimer disease
16	090889	82	--	4.00	Uraemia	Alzheimer disease
17	060290	89	--	3.45	Pneumonia	Alzheimer disease
18	071390	66	--	3.45	Lung infection	Alzheimer disease
19	052990	85	--	3.45	Bronchopneumonia	Alzheimer disease
20	071888	81	--	3.45	Pneumonia/Heart disease	Alzheimer disease
21	121387	88	--	3.45	Decompensatio cordis	Alzheimer disease
22	071490	84	--	3.35	Decompensatio cordis	Alzheimer disease
23	051490	85	--	3.30	Cachexia	Alzheimer disease
mean:		82		3.54		
Non-neurological disease controls						
24	060890	83	--	8.00	Bronchopneumonia	
25	111990	73	--	6.35	Heart failure	
26	111990	85	--	6.34	Heart failure	
27	061990	76	--	6.27	Lung carcinoom	
28	052990	82	--	5.20	Urotheel carcinoom	
29	082890	84	--	4.25	Pancreatic carcinoom	
30	100490	57	--	4.25	Hart failure	
31	070290	74	--	3.45	Cardial infarct	
mean:		77		5.41		

Table 1
Relevant autopsy data as stated in patients autopsy reports of The Netherlands Brain Bank in Amsterdam, The Netherlands (coordinator Dr. R Ravid), were ranked according to PMD.
[a] age in years; [b] post mortem delay in hours; [c] not known.



Photograph 1
Anti-myelin basic protein antibody forming cells were stained with a MBP-peroxidase conjugate. An antibody forming cell specific for myelin basic protein is shown in a cluster of inflammatory cells. Original magnification 500x. Bar is 5 μm.

Discussion

In this study, we present new and direct evidence for the presence of, putative pathogenic, autoantigen specific AFCs in CNS tissue sections of MS patients. To this end, the immunohistochemical technique making use of antigen-enzyme conjugates, which was originally developed for the detection of AFCs in lymphoid tissues (for review: Claassen et al., 1992), was made applicable for the detection of antigen specific AFCs in CNS tissues as well. Making use of MBP-PO conjugates, we have detected MBP specific AFCs in cerebellum and periventricular white matter tissue sections of MS patients. The presence of anti-MBP AFCs in CNS tissues is highly specific for MS patients, no anti-MBP AFCs were detected in a panel of control CNS tissues of patients with other neurological diseases or healthy subjects.

In situ detection of anti-MBP AFCs in only a part of the panel of MS patients is in agreement with *in vitro* studies performed by Cash (Cash et al., 1992). Several reasons might explain why MBP specific AFCs were not detected in all individual samples of the panel of MS patient CNS tissues tested. The most likely reason, is probable the large PMD by which a substantial part of the tissues was sampled. It is a well known phenomenon that a long PMD and a relatively high body temperature of the patients body during the period between decease and autopsy have a negative influence on the quality of the tissues and as a consequence might influence the

Table 2
Frequency Myelin Basic Protein specific AFCs in brain tissue.

Code	Tissue	Plaques[a]	MBP-Specific AFCs[a] Individual	Cuffs	Clusters	Total	Total Ig-AFCs[a]	Number of Clusters[a]
MS patients								
1	cerebrum	+	0	0	0	0	580	13
	cerebrum	-	0	0	0	0	0	1
2	cerebrum	+	0	0	0	0	102	147
	cerebrum	-	0	0	0	0	40	58
3	cerebrum	+	0	0	8	8	266	60
4	cerebellum	-	0	0	0	0	4	0
5	cerebrum	+	0	0	0	0	208	28
	cerebrum	-	0	0	0	0	65	0
6	cerebrum	+	0	0	0	0	0	0
	cerebrum	-	0	0	0	0	0	0
	cerebellum	-	0	0	0	0	0	0
7	cerebrum	+	13	5	5	23	766	20
8	cerebrum	+	2	7	9	18	128	230
	cerebrum	-	7	1	4	12	66	140
9	cerebrum	+	12	2	81	95	206	220
	cerebrum	-	17	0	5	22	314	1190
10	cerebrum	+	1	0	9	10	67	160
	cerebrum	-	2	0	21	23	88	270
	cerebellum	-	2	0	4	6	12	30
11	cerebellum	-	0	0	0	0	534	24
12	cerebellum	-	0	0	0	0	43	16
OND[b] patients (n=11)								
	cerebrum	-	0	0	0	0	2 (2.8)[c]	0
	cerebellum	-	0	0	0	0	30 (27)[c]	0
Non-neurological controls (n=8)								
	cerebrum	-	0	0	0	0	12 (11)[c]	0
	cerebellum	-	0	0	0	0	3 (3.6)[c]	0

The level of inflammation in the tissues was evaluated by counting the number of peri-venous inflammatory cell clusters. A cell cluster is defined as an individual local accumulation of 10 cells or more. The frequency AFCs and number of clusters in CNS tissue sections is expressed per 10 cm^2. The total MBP specific AFC frequency is the enumeration of the three separate frequencies of MBP specific AFCs, detected in cell clusters, peri-venous cuffs and individual respectively.
[a] per 10 cm^2; [b] other neurological diseases; [c] standard deviation.

Table 3
Relative frequency Myelin Basic Protein specific AFCs in brain tissue.

Code	Tissue	Plaque	Relative frequency specific AFCs			
			Individual	Cuffs	Clusters	Total
MS patients						
1	cerebrum	+	0	0	0	0
	cerebrum	-	0	0	0	0
2	cerebrum	+	0	0	0	0
	cerebrum	-	0	0	0	0
3	cerebrum	+	0	0	3	3
4	cerebellum	-	0	0	0	0
5	cerebrum	+	0	0	0	0
	cerebrum	-	0	0	0	0
6	cerebrum	+	0	0	0	0
	cerebrum	-	0	0	0	0
	cerebellum	-	0	0	0	0
7	cerebrum	+	6	2	9	17
8	cerebrum	+	0	4	10	14
	cerebrum	-	4	2	12	18
9	cerebrum	+	5	3	38	46
	cerebrum	-	5	0	2	7
10	cerebrum	+	3	0	12	15
	cerebrum	-	6	0	20	26
	cerebellum	-	2	0	50	52
11	cerebellum	-	0	0	0	0
12	cerebellum	-	0	0	0	0
OND[a] patients (n=11)						
	cerebrum	-	0	0	0	0
	cerebellum	-	0	0	0	0
Non-neurological controls (n=8)						
	cerebrum	-	0	0	0	0
	cerebellum	-	0	0	0	0

The relative frequency MBP specific AFCs in brain tissue was determined, by calculating the frequency of MBP specific AFCs as a proportion of all IgG producing AFCs in serial sections.
[a] other neurological diseases.

detectability of AFCs. In half of MS patients the PMD to tissue sampling is excessive. Most CNS tissue samples of MS patients in which anti-MBP AFCs were detected, were frozen within 10 hours, whereas the tissues of most MS patients in which no anti-MBP AFCs could be detected or in which only small numbers of AFCs could be detected, were sampled with larger PMDs (table 1, 2). A more careful acquisition of tissue, i.e. tissue sampling within 10 hours in subsequent studies, will probably improve the results.

A second reason why MBP specific AFCs were not detected in the whole panel of CNS tissues tested, might be due to the tissue section sampling method. Because the non plaque containing sections were sampled *ad random*, it is possible that a part of the sampled CNS tissues did not show local inflammation and AFCs directed to putative CNS antigens.

CNS tissue sections of MS patients were only tested on the presence of anti-MBP AFCs. However, it is possible that anti-MBP AFCs will not be formed during the entire course of the disease at all. In these cases, the causative antigens may be other CNS component antigens. In subsequent studies, CNS tissue sections of MS patients will be tested on AFCs with other specificities like proteolipid protein and myelin oligodendrocyte glycoprotein.

Furthermore, we only used tissue samples obtained by autopsy, the patients stage of disease at the time of decease may determine whether or not specific AFCs will be detected. Warren and Catz (1986) showed that antibodies in the CSF of MS patients are especially present during exacerbations of clinical signs. Therefore, it is possible that the MS patients, in which we could not detect anti-MBP AFCs, did not show clinical exacerbations prior to the moment of decease.

We have detected anti-MBP AFCs in both plaque containing and non-plaque containing CNS tissues of MS patients. Although the results do not exclude a putative role for MBP specific cells in the not yet affected areas of the CNS of MS patients, the presence of anti-MBP AFCs in both affected and non-affected areas, suggests that the anti-MBP AFCs are directed from the non-affected areas towards the affected areas. Once arrived in the affected areas the anti-MBP AFCs may play a role in demyelination and remyelination as well. The AFCs could function in at least three ways. First, the AFCs may function as antigen presenting cells to activated T-cells. Although antigen specificity is not a requirement for AFCs to act as an antigen presenting cell, the antigen specific anti-MBP AFCs present antigen with a higher efficiency to T-cells as compared to non-antigen specific AFCs (Lanzavecchia, 1985). Since, B-cells are not able to activate resting T-cells (Lassila et al., 1988), the function of AFCs in the process of demyelination, as antigen presenting cell, is restricted to the augmentation of the disease.

Another mechanism by which anti-MBP antibodies and AFCs might be involved in demyelination is the antibody dependent cellular cytotoxicity. Experimental evidence for antibody involvement in cytotoxicity was obtained by Satoh and coworkers (Satoh et al., 1991). They showed that *in vitro* cytolysis of

oligodendrocytes is mediated by human K cells in the presence of anti-oligodendrocyte antiserum or with anti-galactocerebroside antibodies. In addition, serum from MS patients can induce cytotoxicity to rat oligodendrocytes in culture (Ruijs et al., 1990). These results, obtained with *in vitro* experiments, suggests anti-MBP antibodies and AFCs could play a similar role in *vivo* cytotoxic demyelination.

Furthermore, there are some indications for suppressor cell functions of B-cells in the process of demyelination. It is established that sera obtained from recovered EAE animals is able to reduce the incidence and/or severity of the disease when given to recipient animals after induction of the disease (Willenborg, 1981; Killen and Swanborn, 1982; MacPhee et al., 1990). In addition, suppressor cell function is awarded to MBP primed B-cells, which can transfer protection against EAE, together with $CD4^{+}$ T-cells, in naief recipients (Karpus and Swanborn, 1991). Although the evidence for the existence of suppressor B-cells in the process of demyelination is mainly based on data obtained with EAE animal experiments, the results are suggestive for suppressor B-cell function in MS patients.

Several authors have demonstrated the presence of antibodies and AFCs directed against a variety of putative CNS antigens in MS patients. However, the experiments performed sofar concerned the detection of specific AFCs and antibodies in sera and CSF only (Warren and Catz, 1990; 1992b). Because, MBP peptide fragments were detected in both CSF (Whitaker, 1977) and urine of MS patients (Whitaker, 1987), one could still assume that the presence of specific AFCs and antibodies in sera and/or CSF of MS patients is an epiphenomenon due to the release of CNS antigens in the circulation. However, the detection and localisation of anti-MBP AFCs in CNS tissues of MS patients only, as was shown in this study, is very suggestive for a specific immune-pathological role of antibodies and AFCs in MS patients. This implies that methods for an active interference on the level of AFC development, activation or function could open new possibilities for MS therapy development.

Acknowledgement

Tissues were obtained from The Netherlands Brain Bank in Amsterdam (coordinator Dr. R. Ravid). This project is financially supported by the "Foundation Friends Multiple Sclerosis Research", project MS89-50.

CHAPTER 2.6

DISCUSSION
SIGNIFICANCE OF AUTOANTIGEN SPECIFIC ANTIBODY FORMING CELLS IN MULTIPLE SCLEROSIS

The role of B-cell immunity has received relatively little attention in multiple sclerosis (MS) research. One reason for this is that in contrast to T-cells, myelin specific antibodies experimentally can not transfer the disease in animals. In part, this is not surprising since antibodies can not pass the blood brain barrier in healthy animals. Blood born structures with a high molecular weight are generally excluded from the brain and have been found to leak into the brain only at the peak of the experimental allergic encephalomyelitis (EAE) attack (Lam, 1986). However, antibody deficient rats fail to develop clinical or histological evidence of EAE, when sensitized with either whole spinal cord or purified myelin basic protein (MBP) (Willenborg and Prowse, 1983). Intravenous injection of monoclonal antibodies (Mabs) against myelin oligodendrocyte glycoprotein (MOG) in EAE Lewis rat, resulted in augmentation of both clinical signs, inflammation and demyelination (Lassmann et al., 1988). Demyelination also can be induced in central nervous system (CNS) tissue cultures with anti-galactocerebroside serum (Saida et al., 1979). Furthermore, in the presence of anti-MOG Mabs, 10-100 times less encephalitogenic T-cells are needed to induce EAE (Linington et al., 1988). These results indicate that, at least in EAE, the inflammatory process becomes pathologically manifest in the presence of autoantigen reactive antibodies.

Many experiments have been performed to detect and to identify the autoantigen specificity of antibodies and antibody forming cells (AFCs) from patients with MS. The use of classical detection methods allow to reveal the antigen specificity of antibodies produced by isolated cells or present in sera, cerebrospinal fluid (CSF) or tissues only. Applying these classical detection methods, autoantibodies to (MBP) and other myelin proteins, such as proteo-lipid protein, MOG and myelin associated glycoprotein were detected in MS-patients. The actual repertoire of the B-cell, e.g. the produced antibodies, in most studies has been measured by performing direct ELISAs with sera or CSF isolated from MS patient. In these studies only circulating (non-tissue or antigen bound) antibodies, were measured. However it is possible that an essential portion of the autoantigen reactive antibodies is bound, by its Fc portion, to effector cells or with its Fab portion to the target antigen, as a consequence these antibodies will not be measured in body fluids by direct ELISA. Therefore this type of experiments probably provides an incomplete view on the actual B-cell repertoire. Furthermore from these studies no information can be obtained with respect to possible correlations between the local presence of autoantigen specific antibodies or AFCs

and the induction or enhancement of local tissue damage in the CNS.

In our experiments, using a newly developed immuno-histochemical technique, based on the application of autoantigen-enzyme conjugates, we were able to determine the localization of AFCs *in situ* and simultaneously determine antigen specificity of these AFCs. In addition, this approach circumvents problems due to *in vivo* tissue adsorption of the autoantibodies and provides information on the quantity of autoantibodies produced.

Applying immune-histochemistry, we have shown that MBP specific AFCs are present in autopsy brain tissue section of EAE-rhesus monkeys and MS patients. The resemblance of the localization of MBP-specific AFCs in affected CNS tissue sections of both EAE-rhesus monkey and MS patients, makes the EAE-rhesus monkey a useful model for following studies concerning the involvement of B-cells in the process of demeyelination. MBP-specific AFCs were not detected in patients with other non-inflammatory neurological diseases nor in healthy controls, this indicates that auto-reactive AFCs or their produced antibodies may play a role in the process of demyelination in the CNS of MS patients. However, the demonstration of autoantigen specific cells by itself, does not provide information whether these cells or the produced antibodies are involved in the primary or secondary process of pathogenesis of the disease.

Antibodies or antibody forming cells may be involved in the process of demyelination in three ways. First, auto-antibodies might be involved in demyelination by antibody dependent cellular cytotoxicity. It was shown that *in vitro* cytolysis of oligodendrocytes is mediated by human K cells in the presence of anti-oligodendrocyte serum (Satoh et al., 1991). In addition, serum from MS patients can induce cytotoxicity to rat oligodendrocytes in culture (Ruijs et al., 1990). These results suggests that auto-antibody dependent cellular cytotoxicity could play a similar role in the induction or propagation of *in vivo* demyelination. Second, antibodies specific for CNS autoantigens like MBP maybe involved in complement mediated demyelination (Pabst et al., 1971) or in opsonization for specific macrophage mediated cell lysis (Williams et al., 1980). Third, antibody forming cells may function as antigen presenting cells. The recognition and binding of antigens with the membrane bound immunoglobulins is the initial activation signal for B-cells. The immunoglobulin bound autoantigens maybe internalized, processed and subsequently presented in context of MHC class II molecules to T helper (Th) cells. The recognition of the processed antigens in association with MHC class II molecules by the Th-cell receptor will activate Th-cells, which in turn will activate other immunological competent cells. In this way the actual antigen repertoire of the B-cell influences indirectly the T-cell repertoire. The B-cells then function as secondary pathogenic cells.

The hypothesis on a possible B-cell involvement in the pathogenesis of MS, would be supported by the demonstration of the presence of immunological components necessarily for B-cell mediated demyelination in effected areas of the

brain, i.e. effector cells like natural killer, macrophages and T-helper cells for B-cell activation. To investigate this, as described in chapter 3, autopsy CNS tissue sections of MS patients were examined for the presence of T-cells expressing the gp39 activation marker which is the ligand for the B-cell CD40 surface protein. Furthermore we have investigated the role of gp39-CD40 interactions on the development of disease in EAE animals by administration of anti-gp39 monoclonal antibodies.

CHAPTER 3

MODULATION OF EAE BY BLOCKADE OF CD40-CD40-LIGAND INTERACTIONS

CHAPTER 3.1

FUNCTIONAL AND HISTOLOGICAL EVIDENCE FOR CD40-CD40-LIGAND INTERACTIONS IN MULTIPLE SCLEROSIS

K Gerritse[*], JD Laman[*], RJ Noelle[†], A Aruffo[‡], JA Ledbetter[‡],
WJA Boersma[*] and E Claassen[*]

[*]*TNO Prevention and Health, Leiden, The Netherlands*
[†]*Dartmouth Medical School, Lebanon NH, USA*
[‡]*Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle WA, USA*

Submitted for publication

Summary

Activated helper T-cells expressing CD40-ligand surface protein were found in multiple sclerosis patient brain sections, but not in brain tissue sections of normal controls or patients with other neurological diseases. CD40-ligand positive cells were co-localized with CD40 bearing cells in active lesions (perivascular infiltrates or plaques). Most of these CD40 bearing cells proved to be of the monocytic lineage (macrophages, microglial cells), and relatively few were B-cells.

To functionally evaluate CD40-CD40-ligand interactions, experimental allergic encephalomyelitis (EAE) was elicited in mice by means of proteolipid-peptide immunisation. Treatment, with anti-CD40-ligand monoclonal antibody, completely prevented the development of disease.

Furthermore, administration of anti-CD40-ligand MAb, even after disease onset, shortly before maximum disability score was reached, led to dramatic disease reduction.

The presence of Th-cells expressing CD40-ligand in brain tissue of multiple sclerosis patients and EAE animals, together with the functional evidence provided by successful experimental prevention and therapy in an animal model, indicates that blockade of CD40-CD40-ligand, essential for cognate cell-cell interactions, may be considered to be a method for interference in active multiple sclerosis.

Introduction

Many activation signals, surface proteins and co-stimulatory factors are required for the optimal proliferation and differentiation of antigen specific B-cells. Binding of antigens to B-cell membrane bound Ig is followed by internalization and processing of the antigen. Subsequently the antigen is presented in context of MHC class II molecules to T helper (Th) cells. Recognition of this complex by the Th-cell receptor activates Th-cells to express CD40-ligand (Noelle et al., 1992; Armitage et al., 1992) and to produce and secrete cytokines. Binding of the CD40-ligand to its receptor CD40, which is present on all mature B-cells (Clark and Lane, 1991), is required for the initiation of B-cell activation (Paulie et al., 1989). Isolated Th-cell plasma membranes, which express CD40-ligand, can activate resting B-cells *in vitro* (Noelle et al., 1991). The activation of resting B-cells by isolated Th-cell membranes can be blocked both by a soluble CD40-Ig fusion protein and by anti-CD40-ligand monoclonal antibody (Mab). In addition it was shown that CD40-CD40-ligand interactions are essential for thymus dependent humoral immunity (i.e. antibody production) *in vivo* (Foy et al., 1993; Eertwegh et al., 1993).

Although less well described, CD40-CD40-ligand interactions are thought to play a role activation of the monocytic lineage as well (Alderson et al., 1993). Treatment of human monocytes with granulocyte macrophage colony-stimulating factor, interleukin-3 or interferon γ resulted in the induction of CD40 mRNA and enhancement of cell surface CD40 protein expression. CD40 was found to mediate monocyte adhesion to cells transformed to express CD40-ligand. The CD40-ligand transfected cells provided a co-stimulatory signal for monocytes to produce tumor necrosis factor-α and interleukin-6 in the presence of granulocyte/macrophage colony-stimulating factor or interleukin-3 .

Both B-cells and monocytes are considered to play a role in the pathogenesis of multiple sclerosis (MS). Active lesions in the central nervous system (CNS) of MS patients are characterized by mononuclear cell infiltrates. The infiltrating cells comprise T-cells, B-cells and macrophages (Traugott et al., 1982). About 50% of the mononuclear cells in the perivascular lesions in the CNS of experimental autoimmune animals (EAE), an animal model considered to represent the effector phase of MS, are blood-borne monocytes/macrophages (Huitinga et al., 1990) and microglial cells (Bauer et al., 1994; De Simone et al., 1995). In Lewis rats, in which EAE was induced either by adoptive spleen cell transfer or by active immunization with CNS white matter homogenate, the elimination of macrophages suppressed the development of EAE neurological signs significantly (Huitinga et al., 1992). This suggests an important role for macrophages in EAE.

Antibody producing B-cells and antibodies specific for a variety of putative CNS antigens like cerebellar soluble lectin (Zanetta et al., 1990b), glycolipids (Endo et al., 1984), myelin basic protein (Link et al., 1990) (MBP), proteolipid protein (PLP) (Sun et al., 1991) and myelin associated glycoprotein (Baig et al., 1991) have

been detected in both cerebrospinal fluid and sera of MS patients respectively. Recently, we have detected autoantigen specific B-cells in cerebellum and cerebrum tissue sections of MS patients as well (Gerritse et al., 1994). Anti-MBP specific B-cells were detected in CNS tissue sections of MS patients only and not in CNS tissue sections of patients with other neurological diseases nor in CNS tissue sections of healthy controls. However, it is not clear whether these antigen specific B-cells are activated by Th-cells in the demyelinated areas of the CNS or in peripheral localized lymphoid tissues. In order to investigate whether CD40-ligand bearing Th-cells are present in affected areas of MS patient CNS tissues, we have examined CNS tissue section on the presence of CD40-ligand bearing Th-cells using immunohistochemistry in the present study.

In view of the above literature and our finding of CD40-ligand positive T-cells in human MS brain material, we investigated whether the interactions of T-cell activation markers and their ligands, such as CD40-ligand/CD40 might play a functional role in the initiation or development of the disease process in EAE. To this end the *in vivo* interaction between Th-cell activation marker CD40-ligand and its receptor CD40 was blocked by administration of anti-CD40-ligand antibody prior to onset or during progression of EAE (fulminant disease). EAE mice were treated in several time- and dose-administration regimens with anti-CD40-ligand Mabs. The effect of anti-CD40-ligand Mab treatment on the course of the disease was monitored by evaluation of EAE clinical/disease characteristics (disability score), body weight reduction and by determination of serum anti-PLP peptide antibody responses by standard direct ELISA (Zegers et al., 1991).

Methods

Histochemistry

Human autopsy CNS tissues of MS (n=12), normal controls and other neurological disease (OND) controls were obtained from the Multiple Sclerosis/Control Brain Bank (supported by the Foundation Friends MS Research), Coordinator Dr. R. Ravid, of the Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ, Amsterdam. All histochemical analyses, of both human and mouse tissues, were performed on cryosections (8 micrometers) from snapfrozen material. In a pilot study selected CNS tissues of MS-patients (n=4) were used in which anti-MBP specific B-cells (putatively bearing CD40) were detected on a previous occasion (Gerritse et al., 1994).

CD40-ligand$^+$ bearing cells were detected immunohistochemically in human tissue using a CD40-Ig fusion protein as described in detail before (Van den Eertwegh et al., 1993) and in the mouse with the monoclonal hamster antibody MR1 (Noelle et al., 1992; Van den Eertwegh et al., 1993), directly labeled with alkaline phosphatase. Alkaline phosphatase was revealed, in blue, as described before (Van Rooijen et al., 1989), briefly: 8 mg naphthol-AS-MX-phosphate (N-4875, Sigma, St.

Louis, USA) was dissolved in 400 μl DMF and added to 65 ml Tris-HCl buffer (0.1M, pH 8.5). Sixteen mg fast blue BB base (F-3378, Sigma) was suspended in 400 μl 2N HCl. Sixteen mg $NaNO_2$ was dissolved in 400 μl MQ water and added to the fast blue BB base solution. This solution was mixed for 1 min and added to the Tris-HCl buffer. Sixteen mg levamisole was dissolved in 1 ml Tris-HCl buffer and added to the substrate solution. After 2 min incubation at 37°C in a waterbath the substrate solution was filtered to remove any precipitate. Slides were incubated vertically in coplin jars for 10-45 min at 37°C in a waterbath. CD40 was revealed with a mouse anti-human CD40 antibody (5D12; a kind gift of Dr. M de Boer, Pangenetics, Heemstede, Netherlands, De Boer et al., 1993), followed by rabbit-anti-mouse-horseradish peroxidase; RAMPO, P-260, DAKO, Denmark) and immunochemistry with 3-amino-9-ethylcarbazole (AEC, A-5754, Sigma) for a red color. Briefly: 26 mg AEC dissolved in 1.5 ml NN-dimethylformamide was added to 65 ml sodium-acetate buffer (0.05 M, pH 5.0), just before use 35 μl 30% H_2O_2 was added. Slides were incubated vertically in coplin jars for 8 minutes, reaction was ended by transferring the slides to PBS. CD40 was revealed in a blue color after incubation with MAb 5D12 followed by horse-anti-mouse-biotin (Vector, Burlingame, USA) and subsequent application of streptavidin-alkaline phosphatase (Gibco-BRL, Gaithersburg, USA; for alkaline phosphatase see above). Cells of the monocytic lineage (Eikelenboom, 1978; Frei et al., 1987; Ulvestad et al., 1994) were detected, in red, by acid-phosphatase staining (demonstrating endogenous enzyme activity in lysosomes, Van Rooijen et al., 1989). Briefly: 20 mg naphthol AS-BI phosphate (N-2250, Sigma) was dissolved in 2 ml NN-dimethylformamide and mixed with 10 ml barbital buffer (0.15M pH 10) and 24 ml aqua dest. 1.6 ml hexazotized pararosaniline (Sigma) was mixed with 1.6 ml 4% $NaNO_2$ solution and added to the barbital-substrate solution. The solution was brought to pH 5.0 with 1N HCl or 1N NaOH. Slides were incubated horizontally at 37°C for 30 minutes (Van Rooijen et al., 1989). CD11b (complement receptor 3, Frei et al., 1987; Ulvestad et al., 1994) was revealed after incubation with Leu-15 (Becton Dickinson, CA, USA) followed by RAMPO and AEC in red. B-cells were demonstrated as described before (gerritse et al., 1994) with mouse-anti-human IgG/IgM directly labeled with horseradish peroxidase and revealed with AEC in red.

EAE induction and anti-gp39 injection

EAE was induced according to a standard protocol in three groups (n=18) of female SJL/J mice (age 12-15 weeks) by two subcutaneous injections of 75 μg, 150 μg or 300 μg PLP-peptide in the abdominal flanks. The PLP-peptide being amino-acid sequence 139-151 of rat PLP (Dautigny et al., 1985). Peptide synthesis was performed using f-moc amino-acids with the 9500 Milligen synthesizer according to standard protocols. The standard EAE induction procedure using this peptide, results in the development of acute EAE, clinically and pathologically identical to EAE induced using whole CNS myelin or MBP (Tuohy et al., 1989; Sobel et al.,

1990). The emulsion contained per 100 μl: 25 μg *Mycobacteria tuberculosis* (H37RA, Difco) in 50 μl FCA and 37.5 μg, 75 μg or 150 μg peptide in 50 μl PBS. On day 0 and 2, each mice was injected intravenously with 200 μl *Bordetella pertussis* suspension (10.10^{10} /ml PBS). Mice were injected intraperitoneally with 125 μg hamster anti-gp39 Mabs (Noelle et al., 1992; Van den Eertwegh et al., 1993) in 200 μl PBS on days [0, 2 and 4], [4, 6 and 8] or [7, 9 and 11]. Mice of the control groups received 125 μg normal hamster antibodies (Serva, Feinbiochemica) in 200 μl PBS. The severity of EAE clinical signs was evaluated each day and graded according to discrete criteria: Disability scale (DAS) units; grade 0 = no clinical signs, grade 1 = tail weakness, grade 2 = mild paraparesis and ataxia of the hind legs, grade 3 = severe paraparesis or ataxia of the hind legs, grade 4 = moribund, grade 5 = death due to EAE. Blood samples were obtained on days 4, 9, 14, 21, 31 and 40 by tail vein puncture. Sera were screened in a direct ELISA on the presence of anti-PLP-peptide antibodies.

ELISA

PVC round bottom microtiter plates (Falcon) were coated with PLP-peptide (10 μg/ml in phosphate buffered saline (PBS), 25 μl/well) overnight at 4°C. Subsequently plates were pre-incubated for 30 min at 25°C with gelatine (0.5 mg/ml PBS) to prevent non-specific antibody binding. The plates were washed five times with PBS-G (0.1 mg gelatine/ml PBS) and incubated for 60 min at 25°C with serum dilutions. The plates were washed (5x) with PBS-G and incubated for 1 hr at 25°C with alkaline phosphatase labelled rabbit anti-mouse Ig (Dakopatts, Denmark) in PBS-G. After the plates were washed (5x) with PBS-G, 50 μl of a para-nitrophenyl-phosphate (Boehringer, Germany) solution (1 mg/ml 10 mM diethanol-amine, 1 mM $MgCl_2$, pH 9.8) was added to the wells. After incubation for 30 min at 25°C the absorption was determined at 405 nm.

Results

Detection of CD40-ligand$^+$ Th-cells

As figure 1a shows, CD40-ligand$^+$ cells were detected in CNS tissue sections of MS patients (n=12). Cells were shown to be of the Th phenotype by double staining for CD4 (data not shown). In control CNS-tissue sections of normal individuals (n=5) or of Alzheimer patients (n=5), no cells stained with CD40-Ig fusion protein were observed (1b). Similar CD40-ligand bearing cells could be demonstrated in cryosections of EAE mice with either CD40-Ig fusion protein (crossreactivity of CD40-Ig and MR1 in mouse-immunochemistry was shown before, Van den Eertwegh et al., 1993) or the anti-CD40-ligand antibody MR1 (1c, day 12 after induction of disease). CD40-ligand bearing cells were found in white matter lesions and not outside these lesions nor in control animals.

Detection of CD40 bearing cells

In figure 1a-i serial sections of a representative perivascular infiltrate in a cryosection of MS-brain are shown. From figure 1d it is clear that a majority of the infiltrating cells bears CD40. From our previous study on anti-MBP antibody production in MS brain (Gerritse et al., 1994) we would have expected these cells to be CD40 bearing B-cells, however, only few cells (10-20%) were B-cells. Staining for either acid phosphatase (1f-g) and CD11b (1h) showed that most CD40 bearing cells belonged to the monocytic lineage. Only very few of these cells were detected in control cerebrum tissue sections.

Prevention of EAE by anti-CD40-ligand Mab

Administration of PLP peptide resulted in a dose dependent (more intense with increase of peptide dose) induction of severe EAE. After EAE induction with 75 μg or 300 μg PLP-peptide (data of 150 μg not shown), control mice, or those receiving irrelevant hamster antibodies, showed a significant bodyweight reduction (25%-30%) from day eleven until day seventeen (figure 2, 3, upper panels, shaded bars), the first clinical signs of EAE becoming apparent on day eleven.

Severe disease (most animals moribund or dead) was induced with all peptide doses. The highest average DAS score of the control groups was 3.6 observed on days 16 - 23 (figure 3, middle panel) and a score of 2.3 was observed on days 15 - 22 (figure 2, middle panel) in animals in which EAE was induced with 300 μg or 75 μg PLP peptide, respectively. Strikingly, no bodyweight reduction was observed in animals which were treated on days 0, 2 and 4 with 125 μg anti-CD40-ligand Mab, irrespective of the peptide dose used for disease induction (figure 2, 3, upper panels, black bars).

Even more dramatic, MR1 treated animals showed only minimal or no clinical signs at both doses of PLP-peptide used for EAE induction (figure 2, 3, middle panels, note: no or only very low black bars). Clinical signs in the anti-CD40-ligand treated mice were only found when EAE was induced with the highest dose (300 μg) PLP-peptide, and disappeared on day 31, whereas clinical signs of the control group remained severe (figure 3, middle panel).

In control animals, anti-PLP-peptide serum antibody responses were observed from day 9 until day 40, with maximum responses on day 14 (titer = 1433) and 21 (titer = 2710) after EAE induction with 75 μg or 300 μg PLP-peptide respectively. In contrast, anti-PLP-peptide antibody responses in animals treated with CD40-ligand Mabs on days 0, 2 and 4 were severely delayed and decreased, with highest levels on day 31 (titer = 571) and 40 (titer = 1034) after EAE induction with 75 μg and 300 μg PLP-peptide respectively (figure 2, 3, lower panels).

Figure 1

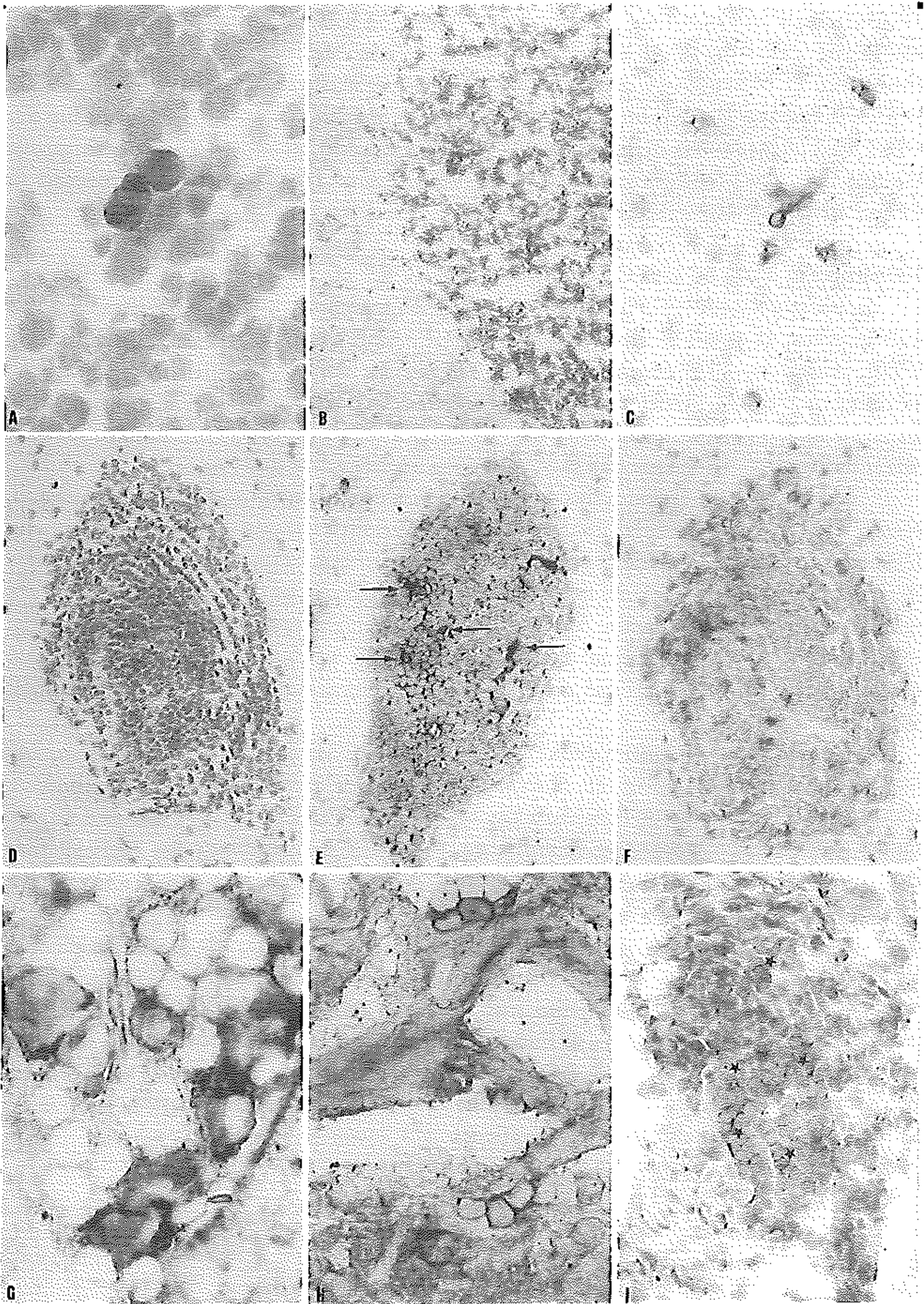


Figure 1 (previous page)
***In vivo* evidence for involvement of CD40-CD40-ligand interactions in EAE and MS.**

A Red: CD40-ligand CD40-ligand positive cells in a perivascular infiltrate (plaque) in human MS-brain. Magnification x 500	B No immuno-stained cells No CD40-ligand positive cells were found in human brain tissues from "normal" controls or other neurological disease (OND). Shown here is a representative section of Alzheimer-brain. Magnification x 50	C Blue: CD40-ligand CD40-ligand positive cells, in a perivascular infiltrate, in mouse brain during EAE. Magnification x 300
D Red: CD40 Numerous CD40 positive cells in a plaque in human MS-brain. Note: D to I are serial sections from the same plaque. Magnification x 100	E Red: IgG/IgM Blue: CD40 Violet: double staining(->) Only few cells positive for CD40 also contain IgG or IgM (arrows). This indicates that only a minority of CD40 expressing cells in the placues belongs to the B-cell subset (10-20%). Magnification x 100	F Red: Acid phosphatase Numerous cells having acid phosphatase activity in lysosomal compartments are present. This indicates that these cells have phagocytic properties and are therefore monocytes/macrophages or microglia. Magnification x 100
G Red: acid phosphatase Blue: CD40 The large majority of cells bearing CD40 on their membrane also have acid phosphatase activity in the cytoplasm. This indicates that CD40 positive cells in plaques are presumably monocytes and/or microglia. Magnification x 1000	H Red: CD11b (CR3) Blue: CD40 Violet: CD11b and CD40 The large majority of CD40 positive cells also express the complement receptor 3. Taken together with the acid phosphatase activity, this indicates that CD40 positive cells in plaques are monocytes or microglia. Magnification x 500	I Red: CD40 Blue: CD40-ligand Cells expressing CD40 and cells expressing CD40-ligand are juxtaposed (arrows). This suggests that CD40-CD40-ligand interactions are ongoing in plaques in human MS brain. Magnification x 200

Effect of anti-CD40-ligand treatment during disease.

Treatment with anti-CD40-ligand Mabs around day 6 and day 9 after EAE induction with 150 μg PLP-peptide still resulted in blockade of disease by 80% and 67% respectively as compared to the complete inhibition (100%) in animals treated with anti-CD40-ligand Mabs around day 2 (figure 4). Of the animals treated with anti-CD40-ligand none died due to EAE. In control animals the first EAE clinical signs (and death) were found already on day 11.

Discussion

This study provides evidence that CD40-ligand-CD40 interactions are involved in development of experimental allergic encephalomyelitis in mice and multiple sclerosis in man. Functionally, treatment of mice with antibodies against CD40-ligand both prevented development of disease (prophylaxis) and dramatically suppressed clinical signs when treatment was started after onset of disease (therapy). Histologically, cells expressing CD40 and CD40-ligand were found in the perivascular infiltrates in the CNS of both EAE-mice and MS-patients, but not in control tissues. Double staining procedures revealed that the majority of CD40-expressing cells in human MS-brain were cells of the monocytic lineage, while a minority belonged to the B-cell subset. Finally, juxtaposition of CD40- and CD40-ligand-expressing cells *in situ* was demonstrated, indicative of ongoing cellular interactions.

The interaction between CD40-ligand on activated $CD4^+$ T-cells and CD40 on B-cells has previously been shown to be indispensable for antibody responses against thymus dependent antigens. CD40 ligation by CD40-ligand induces B-cell proliferation, expression of several other activation markers, isotype switching, antibody production, as well as formation of germinal centers and B-cell memory (reviewed in: Durie et al., 1994). However, recent data indicate that the CD40-ligand-CD40 axis is involved not only in humoral immunity, but in development of some autoimmune diseases as well. For instance, anti-CD40-ligand treatment can prevent collagen-induced arthritis (Durie et al., 1993) and lupus nephritis (Mohan et al., 1995) in mice. These data prompted us to evaluate the functional role of CD40-ligand-CD40 interactions in MS and in EAE as a model for MS.

Figure 2 (next page)

Prevention of EAE induced with low quantity peptide, using anti-CD40-ligand Mab.

EAE was induced with 75μg PLP-peptide in female SJL/J mice (n=6) according to a standard procedure. Animals were treated with anti-gp39 Mabs on days 0, 2 and 4 (black bars). Control mice received normal hamster antibodies on the same days (shaded bars). The effect of anti-gp39 Mab treatment was monitored by determination of the body weight (upper panel) by evaluation of clinical signs (middle panel) and by determination of serum anti-PLP-peptide antibody responses by standard direct ELISA (lower panel).

Figure 2

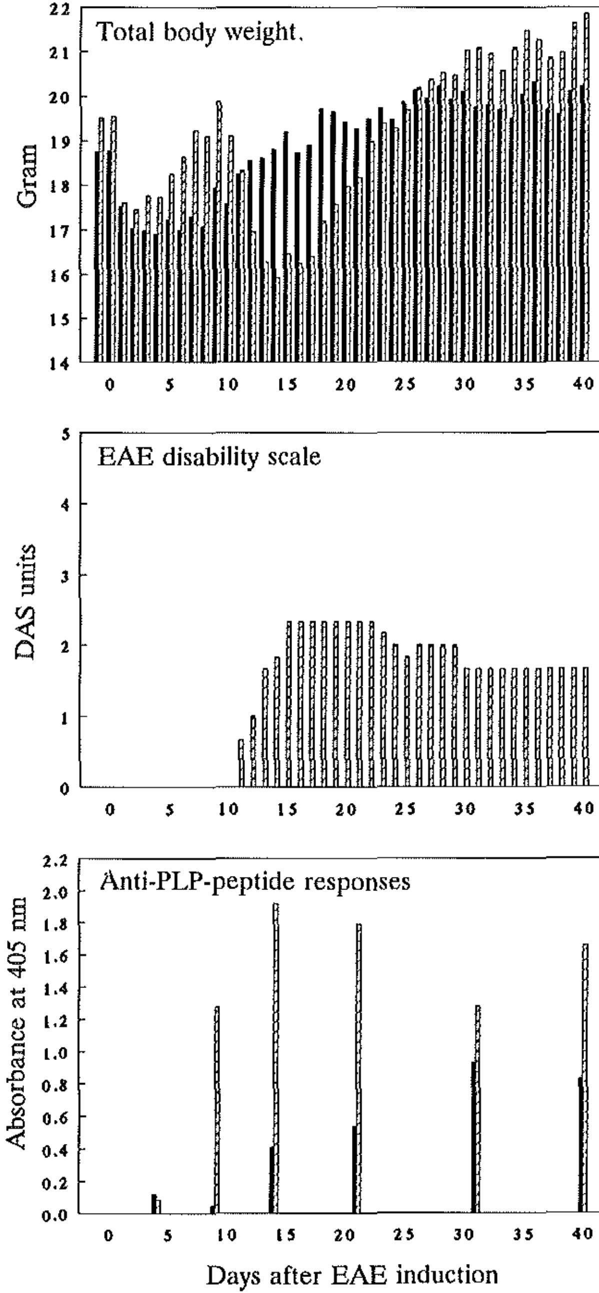
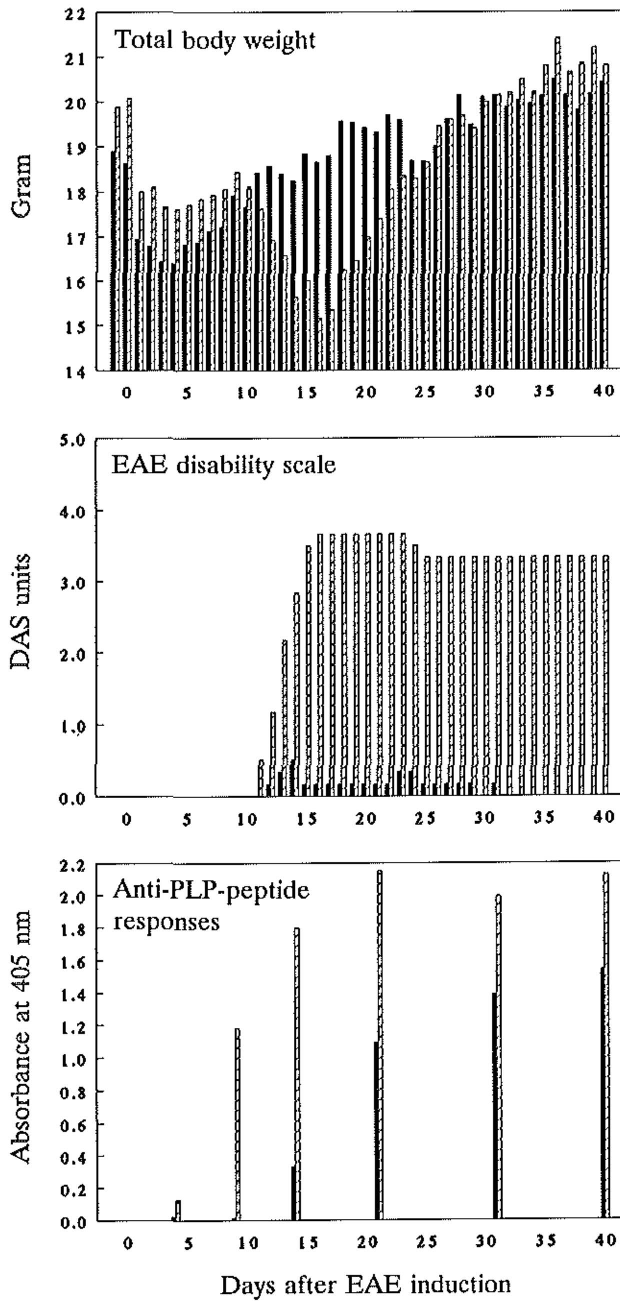


Figure 3



In view of earlier studies on antibody specificity of B-cells (putatively bearing CD40) in MS brain (Gerritse et al., 1994) and considering the functional data obtained in this study, a role for the CD40-CD40-ligand axis in EAE and MS seemed apparent. Consequently, we evaluated whether both these molecules were expressed in EAE and MS. Cells expressing CD40-ligand could be found in perivascular infiltrates of CNS tissue of both EAE mice and MS patients, but not in control tissues. Frequencies of CD40-ligand expressing cells in these infiltrates were modest. This is in accordance with frequencies of CD40-ligand+ cells induced in the spleen by immunization of mice with thymus dependent antigen (TNP-KLH). In this system, approximately one CD40-ligand positive cell was found per 12 KLH-specific B-cells (Van den Eertwegh et al., 1993). In view of the above and the fact that this interaction is absolutely essential for antibody production it is evident that few T-cells can very efficiently ligate with, and trigger, numerous CD40 bearing cells.

Cells expressing CD40 were abundantly present in perivascular infiltrates of MS-brain. Using acid phosphatase and CD11b (CR3) as markers for monocytes/macrophages and microglia (Bauer et al., 1994; Eikelenboom, 1978; Frei et al., 1987; Ulvestad et al., 1994), it was shown that CD40 expression was for the large majority restricted to cells of the monocytic lineage, while B-cells formed a minority of the CD40+ population.

To functionally assess the role of CD40 and its ligand we blocked this cognate interaction *in vivo* by intravenous administration of anti-CD40-ligand antibody. In the EAE-model in mice, treatment with anti-CD40-ligand MAb during disease onset (day 0-4) completely prevented development of disease. Indicating that CD40-ligand-CD40 interactions play an important role in the induction phase of EAE. Development of antibody responses against the PLP-peptide used for immunization was delayed by anti-CD40-ligand treatment, but not completely blocked. This indicates that despite intact B-cell function at later time points after anti-CD40-ligand treatment, EAE does not develop. Either B-cell responses are not crucial to disease induction (as discussed above relatively few B-cells were found in the infiltrates), or B-cells/antibodies become unable to induce disease after a critical susceptible period.

Figure 3 (previous page)

Prevention of EAE induced with high dose peptide, using anti-CD40-ligand Mab.

EAE was induced with 300 μg PLP-peptide in female SJL/J mice (n=6) according to a standard procedure. Animals were treated with anti-gp39 Mabs on days 0, 2 and 4 (black bars). Control mice received normal hamster antibodies on the same days (shaded bars). The effect of anti-gp39 Mab treatment was monitored by determination of the body weight (upper panel) by evaluation of clinical signs (middle panel) and by determination of serum anti-PLP-peptide antibody responses by standard direct ELISA (lower panel).

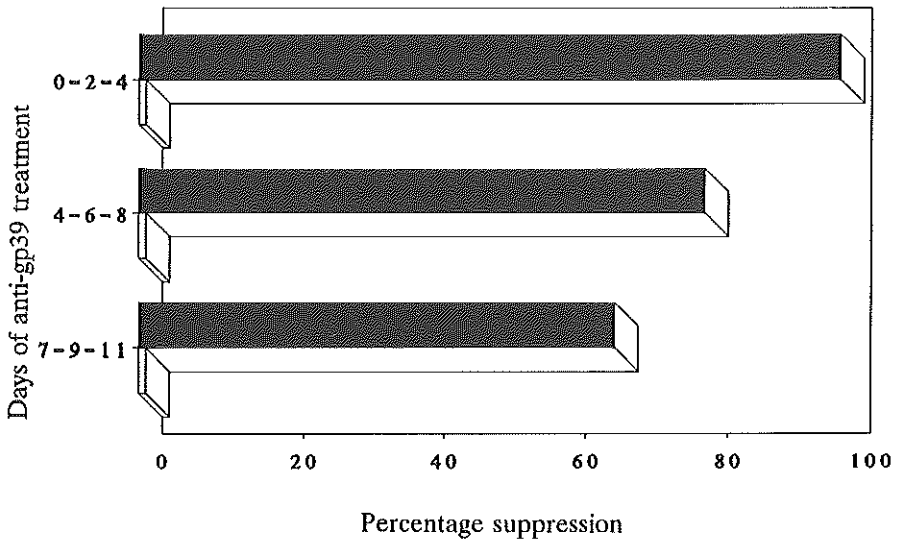


Figure 4
Effect of delayed anti-gp39 treatment on EAE.
EAE was induced with 150 μg PLP-peptide in female SJL/J mice (n=6) according to a standard procedure. Animals were treated with anti-gp39 Mabs on days [0, 2 and 4], [4, 6 and 8] and [7, 9 and 11] (shaded bars). Control mice received normal hamster antibodies on the same days (black bars). The effect of anti-gp39 Mab treatment was monitored by evaluation of clinical signs. The cumulative DAS scores from day 12 until day 28 of mice treated with gp39 on days 0, 2 and 4 was set at 100 % EAE suppression. Cumulative DAS scores from day 12 until day 28 of the other groups were related to this percentage.

Importantly, delayed treatment with anti-CD40-ligand MAb, initiated shortly before maximum clinical disease score is reached, leads to near-total suppression of disease (figure 4). These results indicate that CD40-ligand-CD40 interactions are not only important in the induction phase but in the active phase of EAE as well, thereby providing a target for therapy as well as for prevention. As a consequence, this implicates that the development of this inflammatory disease of the CNS is dependent on continuous or repeated CD40-ligand-CD40 interactions (as shown for humoral responses against "model" antigens; Van den Eertwegh et al., 1993).

What effector mechanisms are induced by CD40-CD40-ligand interactions in the CNS during EAE and MS? The immunocytochemical data discussed above clearly showed that the majority of CD40-bearing cells were macrophages or microglia. In addition, a small subpopulation of CD40+ cells belonged to the B-cell lineage. Consequently, CD40-ligand-induced functions of both macrophages/ microglia and B-cells should be considered. With respect to B-cells, antibodies specific for or crossreactive with myelin components MS may be involved in the

process of demyelination in EAE, inflicting direct damage or acting through alternative mechanisms, like antibody dependent cellular cytotoxicity (ADCC). It has been shown that sera from guinea pigs with EAE can induce or enhance the breakdown of the myelin sheath, which surrounds the neuronal axons, in the CNS of syngeneic recipients (Bourdette et al., 1986). In addition, intravenous injection of anti-myelin oligodendrocyte glycoprotein MAbs in Lewis rats with EAE resulted in augmentation of both clinical signs, inflammation and demyelination (Lassmann et al., 1988). Willenborg and coworkers (1986), have also demonstrated a role of B-cells in initiation of disease. We have previously found limited numbers of MBP specific plasma cells in the CNS of MS-patients and EAE-rhesus monkeys (Gerritse et al., 1994). However, in view of the relatively limited (when related to the total number of infiltrating cells) number of CD40+ B-cells in lymphoid infiltrates of MS-brain, the anti-PLP antibodies found even after anti-CD40-ligand treatment, and the convincing evidence that EAE can be adoptively transferred by T-cells, but not by B-cells (Ben-Nun and Cohen, 1982; Swanborg, 1983), a role for B-cells in development of disease seems unlikely at present.

This leaves macrophages/microglia (CD11b+, acid phosphatase containing cells: see figure 1d-h) in perivascular infiltrates as the CD40-bearing population crucial to disease development. Consistent with this possibility, Huitinga et al. (1990) have elegantly demonstrated that macrophages are required for development of EAE in rats. What macrophage effector mechanisms contributing to inflammation and/or demyelination in the CNS may be activated trough CD40 triggering? Alderson et al. (1993) have shown that CD40 triggering of human monocytes induced tumoricidal activity, and in presence of appropriate cytokines, TNF-alpha, IL-6 and IL-8 was produced. In addition, Wagner et al. (1994) found IL-1 after CD40 triggering, and production of IL-12 by human monocytes was reported by Shu et al. (1995). Finally, Tian et al. (1995) demonstrated that murine splenic macrophages synergize with cytokines to enhance nitric oxide (NO) production. It is obvious that these compounds may directly contribute to inflammation and neurological dysfunction. In this respect it is of note that antibodies against IL-12 prevent development of EAE in mice. It remains to be determined which of these compounds are actually produced *in vivo* in response to CD40-CD40-ligand interactions, and what their relative contributions to disease development are.

How does treatment with anti-CD40-ligand antibody prevent disease development and suppress established disease? As we discussed before (Foy et al., 1994), anti-CD40-ligand does not induce unresponsiveness by a direct cytotoxic effect on T-cells. This notion is supported by findings that anti-CD40-ligand treatment does not delete T-cells, nor does it affect their helper function. In addition, anti-CD40-ligand treatment does not affect the frequencies of IL-2, IL-4 and IFN-gamma producing cells *in situ* (Van den Eertwegh et al., 1993). Collectively, these observations and the current study suggest that amelioration of EAE by anti-CD40-ligand treatment may result from direct blocking of interaction of CD40-ligand on

activated T-cells with CD40 on monocytes/macrophages and microglial cells.

Alternatively, anti-CD40-ligand antibody may induces Th-cell unresponsiveness in EAE by interference with the CD40 related expression of co-stimulatory molecules on antigen presenting cells, resulting in impaired antigen presentation. Although speculative at this time, the results of other studies support this hypothesis in view of the fact that the blocking of interactions between B7 family members and their ligands *in vivo* induce a state of allo-specific T-cell unresponsiveness (Tan et al., 1993). Therefore, receptors like CD40, of which the triggering was shown to regulate the expression of B7.1 and perhaps B7.2 (De Simone et al., 1995; Tan et al., 1993) might play an important role in controlling tolerance and immunity.

Although further details of the mechanism(s) of action are clearly needed, the co-localization of CD40-bearing macrophages/microglia and CD40-ligand-bearing cells in affected CNS tissue of patients suggests that CD40-CD40-ligand may play an important role in the immunopathology of multiple sclerosis. In analogy to the results obtained in EAE, blockade of CD40-ligand-CD40 interactions should be considered as a method to interfere in MS as well. Preventing CD40-ligand-CD40 interactions is potentially useful in limiting duration, intensity and neurological damage of disease exacerbations. A significant advantage of CD40-ligand as a target for intervention is its transient expression restricted to activated $CD4^+$ T-cells. This feature allows targeting of only those T-cells actively participating in the response, and not affecting the population of T-cells at large.

Acknowledgements

MS-autopsy brain tissues were obtained from the Netherlands Brain Bank in Amsterdam (coordinator Dr. R. Ravid). We thank Dr. M. de Boer (Pangenetics BV) for supplying the 5D12 MAb directed to human CD40. We thank Marjan van Meurs and Louis Ribbens for excellent histochemical support. This study was financially supported by the Dutch Foundation Friends Multiple Sclerosis Research, project MS89-50, MS94-171 and partially supported by a pilot grant from the National Multiple Sclerosis Society and National Institutes of Health grant AI26296.

CHAPTER 4

ORAL DELIVERY OF MS-AUTOANTIGENS BY *LACTOBACILLUS* TO INDUCE TOLERANCE

CHAPTER 4.1

THE USE OF *LACTOBACILLUS* AS CARRIER FOR THE ORAL DELIVERY OF ANTIGENS

Introduction

Live transformed microorganisms can be applied as antigen carrier for the oral delivery of antigens, provided that these organisms are able to carry the antigen to the proper sites of antigen presentation. The presentation of antigens to the immune system via the oral route may result in either the induction of an immune response or in immunological non-responsiveness. In other words, due to the oral delivery of antigens by microorganisms, the individual will be vaccinated, i.e. a cellular and/or humoral immune response will be developed upon a subsequent presentation of the same antigen, or the individual will be tolerized against the administered antigens. This chapter summarizes the basic understandings concerning oral vaccination and tolerization and describes the principles of the use of microorganisms as antigen delivery system. The use of *Lactobacillus* as a live antigen carrier device will be compared to other microorganisms as potential antigen carriers.

The mucosal immune system

The mucosal immune system comprises the lymphoid tissues which are associated with the gut (GALT, gut associated lymphoid tissues), the respiratory tract (BALT, bronchus associated lymphoid tissues) and several other organs with mucosal surfaces like the salivary- and lacrimal gland and reproductive system. In general the mucosal immune system is the first protection system against environmental pathogens. The local mucosal immunity is relatively independently regulated from the systemic immunity. While the systemic immune response is characterized by antibodies of the IgM and IgG classes, the mucosal immune response is characterized by the production of IgA, which represents more than 60% of all antibody isotypes produced (Mestecky and McGhee, 1987). The IgA immunoglobulins are released along the mucosal surfaces and bind to foreign antigens. The exposure of an antigen at one mucosal site leads to immunological protection after a renewed exposure to the same antigen even when presentation occurs at a distant mucosal surface. Therefore it has been postulated that all mucosal surfaces form an integral specialized lymphoid system.

The intestinal mucosal immune system spans a large surface area and comprises the Peyer's patches and lymphocytes in the lamina propria. An ingested antigen will only be presented to the intestinal mucosal immune system after it has

crossed the intestinal epithelial cells. Several methods of intestinal antigen uptake can be distinguished. I) A non-specific antigen uptake is possible via disrupted tight junctions between epithelial cells, this route of antigen uptake especially occurs in a damaged gut. II) Non-specific antigen uptake can occur at the tip of the microvilli on the epithelial cells due to endocytosis. III) Some epithelial cells, the microfold (M) cells, are especially adapted for IgA mediated transport of antigens from the intestinal lumen to the intestinal lymphoid cells. The M cells can facilitate the transport of both antigens in solution and particulate antigens. Antigen uptake of M-cells does not result in degradation, but instead in delivery of intact antigen to lymphoid cells (Wolf and Bye, 1984). IV) Receptor mediated specific antigen uptake is possible, but for a limited number of antigens only, e.g. cholera toxin.

Oral tolerance

Ingested food antigens in healthy subjects usually do not evoke an immune response but achieve the opposite effect, i.e. the individual will become tolerant to the ingested antigens. The term oral tolerance refers to a state of systemic unresponsiveness to parenteral immunization which is induced by previous antigen feeding. Several mechanisms have been proposed to account for this peripheral unresponsiveness: clonal deletion (Jones et al., 1990), clonal anergy (Whitacre et al., 1991) and specific suppression (Lider et al., 1989).

Oral tolerance for both humoral and cellular immunity has been demonstrated in animals fed with several antigens (Kagnoff, 1978; Challacombe and Tomassi, 1980). Furthermore it has become clear from studies with experimental autoimmune diseases like collagen induced arthritis (Nagler-Anderson et al., 1986), experimental autoimmune uveitis (Nussenblatt et al., 1990) and experimental autoimmune encephalomyelitis (EAE) (Higgins and Weiner, 1988) that these experimental diseases could be prevented, delayed or decreased by feeding antigens prior to disease induction with the same antigen. These results support the idea that it might be possible to exploit oral tolerance in the treatment of human autoimmune diseases as well.

Autoimmune phenomena are considered to play an important role in the pathogenesis of multiple sclerosis (MS). As a consequence, most MS therapies are based on the modification of the immune response. However, apart from pharmaceutical immune suppression of inflammation, most MS therapy trials reported until now were ineffective in the treatment of the disease. Therefore one of the most promising new approaches also for MS therapy might be the oral administration of autoantigens, in order to turn the anti-self (non-tolerance) state into a state of specific tolerance.

From several experiments, in which antigen specific tolerance was induced after oral administration of autoantigens (reviewed in Weiner et al., 1991), some of the conditions to achieve oral tolerance induction as a therapy for human

autoimmune diseases can be derived. Induction of tolerance has been shown to be dependent on I) the antigen dose, II) the duration of antigen administration (Strober et al., 1981; Sjovall and Christensen, 1991) and III) the age of the individual (Van Hoogstraten et al., 1991; Miller et al., 1994). IV) Upon oral administration antigens are degraded to some extent when passing the gastro-intestinal tract. Therefore, autoantigens need to be protected from degradation to be effective (Thompson and Staines, 1990). V) Large quantities of purified human autoantigens would be needed to satisfy the demand for therapy, once adequate regimens have been established.

The use of microorganisms for oral tolerance induction

Thus far in experimental protocols to induce oral tolerance, purified antigens were used which were orally administered in a pulse like fashion by feeding at regular intervals. An ideal therapy however should consist of a continuous production and presence of the autoantigen at the site of tolerance induction, i.e. the GALT. By using genetically transformed live microorganisms as antigen delivery system these demands could possibly be fulfilled. The oral administration and subsequent colonization of microorganisms-autoantigen constructs might realize the continuous presence of an autoantigen producing unit in the gastro-intestinal tract. This offers a number of advantages over traditional oral administration of autoantigens: I) no bulk purification of human proteins is necessary, II) autoantigens are released continuous and not in a pulse way manner, III) therapy can be restricted to a single or only few administrations of the autoantigen producing unit and iv) degradation during the passage of the gastrointestinal tract will be circumvented.

Oral vaccination

The administration of vaccines is generally used as a form of immunoprophylaxis. Vaccination will realize immunological protection even a long time before the organism is exposed to a infectious organism. The processes involved in the generation of the protective properties of the organism after infection are based on two components. The first component, the adaptive system, which include B-cells and T-cells, will replicate, differentiate and maturate only after stimulation with antigen. The second "non-adaptive" component include factors like interferons and complement and cells like macrophages, monocytes and natural killer cells, which are involved in non-antigen specific processes. Since B-plasma and T-effector cells have a short lifespan, the generation of an immunological memory is a necessarily requirement to obtain a continuous protection. The duration of the protection obtained against infectious organisms is dependent on the nature of the antigen. Some vaccines will induce a live long protection while others will induce protection with a restricted duration. Furthermore, the duration of the protection is

dependent on the degree to which an infectious organism is able to change its antigens.

To induce an immunological memory, the above mentioned adaptive and non-adaptive components of the immune system have to be stimulated by administration of the antigen of interest. The antigen can be administered in many way's but of all routes the oral route is the most convenient route of vaccination as compared to the more frequently used parenteral routes, especially in vaccination programs in which large numbers of subjects are involved. One of the advantages of an "oral" vaccine is that the administration needs not to be carried out by a trained medical staff. Furthermore the number of drop outs, those subjects who do not receive their booster injection, will be reduced since the booster vaccine can be provided, together with clear user instructions, simultaneously with the first administration. In addition, special equipment like sterile needles, disinfectants, autoclaves or repeating injectors are not necessary and concomitant risks on co-infections due to the usage of this injection equipment are prevented.

The use of microorganisms for oral vaccination

A newly developed technique to deliver antigens to the immune system for vaccination purposes, is the use of microorganisms as antigen delivery vectors for immunizations. The underlying idea is that by using transformed microorganisms, which expose heterologous proteins, antigens will be delivered to the immune system in such a way that a protective response is triggered against the target pathogen. The strains of microorganisms used are usually genetically attenuated mutants of pathogenic bacteriae (the carrier) transformed to express a genetic construct (the vector) designed to obtain an adequate expression of antigens from other pathogens. Such carriers can then be used as a local antigen producer thereby circumventing expensive purification processes. It is to be expected that the right choice of carriers, vectors and antigens may lead to the desired protective immunity.

Most work done so far involved the use of *Salmonella thyphimurium* (Stabel et al., 1991) and *Salmonella enteriditus* in animals and *Salmonella typhi* (Van de Verg et al., 1990) in man. Attenuated *Salmonella typhi* strains can serve as live oral vaccines to prevent typhoid fever. In an efficiency field trial carried out in Alexandria, Egypt, approximately 32.000 school children were randomized to receive orally three doses of the vaccine (10^9 organism) or placebo. During three years of surveillance a vaccine efficiency of 96% against culture confirmed typhoid fever was recorded (Wahdan et al., 1982). Attenuated *Salmonella* strains have also been used as carrier for hepatitis B virus antigens. Other bacteria which are potentially useful as carrier include strains of *Escherichia* (Hale, 1990), *Mycobacteria*, *Shigella*, *Vibrio cholerae* and *Yersinnia*. However, most transformed microorganism used for oral immunization are recognized as pathogen and their use as antigen carrier may be limited. For this reason the use of a GRAS (Generally Recognized As Save)

organism, such as *Lactobacillus*, as a safe antigen carrier for the delivery of foreign antigens in the gastrointestinal tract of animals and humans is to be preferred.

Lactobacillus

Lactobacillus represents a major genus of the lactic-acid bacteria, a diverse group of microorganisms, that produce lactic acid as the major end product of metabolism. Members of this genus are known, in the first place, for their widespread use in various food and agricultural fermentation processes (Chassy, 1985; 1987) and secondly for its supposed health promoting (probiotic) properties. This makes *Lactobacillus* a very attractive candidate for use as delivery system of antigens by oral administration. In addition, it has been demonstrated that oral administration of specific *Lactobacillus* strains is positively correlated with reduction of serum cholesterol levels (Tannock et al., 1989), detoxification of potential carcinogens (Fernandez et al., 1987; Gorbach et al., 1990), stimulation of the host non-specific immunity (adjuvanticity; Bloksma et al., 1979; Boersma et al., 1994) and inhibition of the outgrowth of microorganisms causing infectious diseases (Perdigon et al., 1986c; 1988). However, the underlying mechanisms for the probiotic properties of *Lactobacillus* have not been explained satisfactory (Pouwels et al., 1992). In addition, *Lactobacillus* can maintain a stable population in both animal and human gut (Perdigon et al., 1986c; 1988; Chassy, 1985; 1987; Gorden and Golbach, 1979; Kleeman and Klaenhammer, 1982), they possess a low intrinsic immunogenicity as compared to other microorganisms (Forrest, 1988; Carlsson and Bratthall, 1985) and Lactobacilli have mucosal adhesive properties (Lencner et al., 1987).

Several genetic transformation procedures for expression of human proteins in Lactobacilli are available. It has become possible to transform virtually any *Lactobacillus* species with plasmid vectors. *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus acidophilus, Lactobacillus fermentum and Lactobacillus brevis* can be transformed routinely with plasmid vectors, making use of the electroporation technique (Posno et al., 1991a; Pouwels et al., 1992). Shuttle-vectors which replicate in both *Escherichia coli* and *Lactobacillus* and vectors which replicate in *Lactobacillus* only, have been constructed previously (Posno et al., 1990; 1991a). All *Lactobacillus* vectors appear to be structurally very stable. With regard to segregational instability it appears, that some *Lactobacillus* vectors are stable while others are not (Posno et al., 1991b; Leer et al., 1992). In addition, vectors have been constructed which allow directed insertion at a specific site in the chromosome, warranting stable maintenance and expression of the cloned gene. This technique also allows the replacement or a directed inactivation of a specific *Lactobacillus* gene (Leer et al., 1993).

These results indicate that *Lactobacillus* strains are potentially useful candidates to be employed as live antigen carriers/producers which can be used as

vaccines or used to induce tolerance after oral administration.

Lactobacillus as antigen carrier

As was described in the preceding section the use of *Lactobacillus* as a live antigen carrier for oral administration offers a number of advantages as compared to the use of other microorganisms as antigen carrier or to traditional feeding of antigens. However, in order to establish the feasibility of the use of *Lactobacillus* as antigen carrier many questions have to be answered. Two prerequisites for the development of *Lactobacillus* as an oral antigen carrier system can be distinguished.

A) The availability of gene-transfer and gene-expression systems for *Lactobacillus* for the introduction and efficient expression of homologous and heterologous proteins of interest. It is of prime importance to develop food grade (stable expression) vectors, since by definition food grade vectors may be applied for use in humans. Food-grade vectors are characterized by a nutritional selection system, homologous DNA and by the absence of an antibiotic resistance marker. As a consequence, the use of Lactobacilli which have been transformed with foodgrade vectors makes it possible to distinguish endogenous from experimental Lactobacilli upon analysis of gut content. Several experiments have already been performed concerning the development of food grade vectors. A number of genes involved in D-xylose catabolism (xyl-genes) in *Lactobacillus pentosus* were cloned and characterized (Lokman et al., 1991). Sequences involved in (regulation) of expression of D-xylose catabolism encoding genes were identified. A *Lactobacillus* expression-vector was constructed by inserting a xyl-gene promoter and a xyl-gene terminator (separated by a multiple cloning site) in one of the *Lactobacillus* vectors previously constructed (Posno et al., 1991b). Furthermore, expression of a heterologous model gene, i.e. *Escherichia coli lacZ* gene, in *Lactobacillus* was established and neutralizing epitopes from FMD virus fused to *Escherichia coli lacZ* were expressed making use of these vectors.

B) Presentation of the appropriate genetically manipulated *Lactobacillus* to the immune system in such way that, dependent on the application, mucosal immunity as well as a systemic memory response is generated or immunological tolerance is induced. Although much work already has been performed concerning the production of antigens by food grade vectors, it is unknown in what way an orally administered *Lactobacillus* strain has to present the antigens to the immune system. There are several methods of antigen presentation possible: 1) the antigen is expressed by the Lactobacilli and secreted in the gut, 2) the antigen is released from the cytoplasm of the Lactobacilli upon death and cytolysis in the gut, or 3) the antigen is expressed on the surface of the Lactobacilli and is presented by either live or dead organisms to the GALT.

When using *Lactobacillus* as antigenic carrier of tolerogenic or immunogenic fragments it is important to reveal the most efficient route of antigen presentation.

For this purpose transformed *Lactobacillus* strains with the above mentioned different antigen presentation systems have to be developed.

In the next chapters we address a number of questions concerning the use of *Lactobacillus* as a new generation of vaccines for infectious diseases. We have determined the efficacy of Lactobacilli-antigen constructs to generate peripheral immunological memory after oral administration (chapter 4.2). In chapter 4.3, we have investigated the localization and routing through the immune system of *Lactobacillus* associated antigens which were orally administered. In addition, in chapter 4.4 we have investigated the possibility to enhance immunological non-responsiveness, making use of oral administered *Lactobacillus* associated antigens.

CHAPTER 4.2

ORAL ADMINISTRATION OF TNP-*LACTOBACILLUS* CONJUGATES IN MICE: A MODEL FOR EVALUATION OF MUCOSAL- AND SYSTEMIC IMMUNE RESPONSES AND MEMORY FORMATION ELICITED BY TRANSFORMED LACTOBACILLI

K Gerritse, M Posno*, MM Schellekens, WJA Boersma and E Claassen

TNO Prevention and Health, Leiden, The Netherlands
**TNO Nutrition and Food Research, Rijswijk, The Netherlands*

Res Microbiol 141:955-962, 1990

Summary

Safe live vectors systems are developed for oral delivery of antigens. For this we developed gene-transfer and expression systems in *Lactobacillus* species. Model systems were set up to evaluate immune responses. Orally administered trinitrophenylized *Lactobacillus* were examined for their ability to induce immunological memory formation via determination of specific antibody titers in serum. We demonstrate a direct correlation between the level of the systemic memory formation, as revealed by specific anti-TNP IgG serum antibodies, and the TNP substitution ratio of the *Lactobacillus* suspension used for oral priming. The specific IgG anti-TNP serum titers were comparable to or even higher than the titers of parental (i.p.) primed animals. These results demonstrate the feasibility of using orally administered antigen-*Lactobacillus* as a future approach to vaccination.

Introduction

Especially in vaccination programs in which large numbers of subjects are involved, the oral route of administration is very convenient as compared to the more frequently used parenteral routes. It has been shown that oral immunization can result in an adequate local expression of IgA responses in various mucosal tissues and secretions (Goldblum et al., 1975; Mestecky, 1987; Nedrud et al., 1987), the systemic antibody response, however, generally appears to be only moderate (even when high doses of antigens are applied). This may partly be due to digestical processing resulting in loss of immunogenic potential of the antigen. When a suboptimal amount of antigen reaches the mucosal immune system an adjuvans is

required to evoke or enhance the immune response. The efficiency of oral immunization may be improved by delivery of antigenic material to the mucosal surface by appropriate antigen carrier systems. It has been well documented that transformed microorganisms can be used as such carriers. However, most of the microorganisms currently used (e.g. *E. coli, S. mutans, S. typhi*; Leclerc et al., 1989; Chatfield et al., 1989) are recognized as pathogens and their acceptibility for practical application may be limited. For this reason, the use of a GRAS (Generally Recognized As Safe) organism as a delivery vector is to be preferred. We have recently started to evaluate oral administration of lactobacilli expressing cloned foreign antigenic determinants as a means of antigen delivery to the gastrointestinal tract. The use of non-pathogenic *Lactobacillus*, a GRAS microorganism offers some major advantages. *Lactobacillus* is able to pass the tractus digestivus and, in some cases, is able to maintain a stable population in the gut (Savage, 1983) probably due to mucosal adhesive properties (Lencner et al., 1987). Furthermore, *Lactobacillus* strains have been shown to possess intrinsic adjuvans activity (Perdigon et al., 1986a), to activate peritoneal macrophages and mononuclear phagocytes (Perdigon, 1986b,c). and they show a low intrinsic immunogenicity (Carlsson and Bratthall, 1985) in comparison with other microorganisms e.g. *Salmonella typhi* (Forrest, 1988).

To develop a safe (non-pathogenic) live vector antigen delivery system we recently initiated investigations on: (1) development of gene-transfer and gene-expression systems for the introduction and efficient expression of antigens in *Lactobacillus* species which are (natural) inhabitants of the gastrointestinal tract and (2) immunological evaluation and optimization of the immune responses (mucosal and systemic) that are generated through presentation of *Lactobacillus*-associated antigens by the oral route.

Material and methods

Transformation procedure

Lactobacillus cells were cultivated in MRS medium (Difco) in the presence of 1% (w/v) glycine (for cultivation of *L.casei* glycine was ommited). Electroporation of lactobacilli was carried out with the "Gene Pulser Controller" from Bio-Rad. Briefly, 1 μg plasmid DNA, cesiumchloride gradient purified, was added to 50 μl of a chilled suspension of (mid-log phase) *Lactobacillus* cells ($\pm$ 10^{10}.ml^{-1}) in 0.3 M sucrose, 5 mM Na-phosphate pH 7.4 and 1 mM $MgCl_2$ in a 0.2 cm-gap disposable cuvet. Electroporation was carried out at 6250 V.cm^{-1} with a parallel 200 ohm resistor and a capacitance of 25 μF. Following the pulse, the cell suspension was directly diluted with 450 μl MRS medium. Transformants were selected by plating the cell suspension on MRS-agar plates containing 10 μg.ml^{-1} chloramphenicol.

Carrier/adjuvans properties of *Lactobacillus*

After aerobic incubation at 37°C overnight the organisms were harvested by centrifugation at 3640 g for 15 min at room temperature and resuspended in PBS ($1x10^{10}$ $CFU.ml^{-1}$). Subsequently the suspension was heat killed for 30 min at 60°C.

In order to obtain different TNP/*Lactobacillus* substitution ratios 1000, 500, 50, 20, and 5 μl of a 2,4,6 trinitrophenyl sulfonic acid (TNBS) solution in PBS (20 $mg.ml^{-1}$) was added to 1 ml *Lactobacillus* suspension (53 $mg.ml^{-1}$ PBS). The suspensions were incubated for 1 h at room temperature in complete darkness, washed 3x and resuspended in 1 ml PBS. The *Lactobacillus*-TNP suspensions were coded according to the quantity of TNBS solution added (table 1). Prior to immunization the suspensions were diluted in 0.2 M $NaHCO_3$ (100 $\mu l.ml^{-1}$) for oral and in PBS (200 $\mu l.ml^{-1}$)for i.p. administration. Trinitrophenylated ovalbumin was used for the detection of anti-TNP antibodies in a direct ELISA. TNBS (100 mg) was added under gentle stirring to 100 mg ovalbumin in 10 ml borate buffer (0.1 M, pH 9.0). The solution was incubated in the dark for 2 h at room temperature, and dialysed against PBS.

Immunization

Lactobacillus-TNP conjugates were orally administered three times on alternating days to groups of four female mice (BALB/c 12-15 weeks old). All oral administrations contained 500 μg *Lactobacillus*, based on dry weight, suspended in 500 μl 0.2 M $NaHCO_3$. Four weeks after the last oral administration, the mice were boosted i.p. with 500 μg TNP-*Lactobacillus* conjugate suspended in 250 μl PBS. Control groups were both i.p. primed and boosted with the same TNP-*Lactobacillus* conjugates as the test groups. Three, five and seven day's after the last oral administration or i.p. prime injection and after the booster injection, blood samples of the mice were collected by bleeding of the tail vein. The sera were screened for anti-TNP and anti-*Lactobacillus* antibody (Ab) reactivity by a direct ELISA (table 1).

Table 1
Immunization scheme.

PRIME								
Immunization route:	oral	oral	oral	oral	oral	oral	i.p.	i.p.
suspensions*:	1000	500	50	20	5	0	50	50
no. of administrations:	3	3	3	3	3	1	1	
BOOST								
Immunization route:	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	i.p.	-
suspensions*:	50	50	50	50	50	50	50	-
no. of administrations:	1	1	1	1	1	1	1	-

* TNP-*Lactobacillus* ratio of the suspensions is indicated in "Materials and methods".

Direct ELISA

Anti-TNP titers were determined in PVC microtiter plates coated with TNP-ovalbumin (5 $\mu g.ml^{-1}$ PBS, 50 μl/well) overnight at 4°C. Non occupied space was blocked with gelatine (5 $mg.ml^{-1}$, 50 μl/well), 30 min at 25°C. After washing with gelatine in PBS (0.1 $mg.ml^{-1}$) the plates were incubated for one hour at 25°C with dilutions of TNP-induced-antisera and pre-immune control sera. Then the plates were washed and incubated for 1 h at 25°C with either alkaline phosphatase labelled goat anti-mouse IgA, IgM or IgG (KPL, Inc., Gaithersburg, USA) and para-nitro-phenyl phosphate (Boehringer, Mannheim, FRG) as the substrate. After 30 minutes the absorption was determined at 405 nm in a Titertek Multiskan (Flow, Irvine, Scotland).

The anti-*Lactobacillus* serum titer was determined on preactivated polystyrene microtiterplates (NUNC). Plates were incubated 2 h at 56°C with glutaraldehyde (0.025% in Na_2CO_3 0.1 M, pH 9.0; 100 μl/well; Baker, grade 25% in H_2O). The plates were washed with gelatine (0.1 $mg.ml^{-1}$ in PBS) and dried for 15 min at 56°C. A suspension of *Lactobacillus* (10 $\mu g.ml^{-1}$ Na_2CO_3 (0.1 M, pH 9.0)) was added to the wells (100 μl/well) and incubated overnight at 4°C. After incubation, the ELISA was performed exactly as was indicated for the TNP-ovalbumin coated microtiter plates.

Results

Gene-transfer system

Most *Lactobacillus* strains have been shown to contain one or more plasmids (i.e. Nes, 1984; Vescovo et al., 1981). We have isolated a 1.8 kb (cryptic) plasmid from *L.plantarum* ATCC 8014 and subsequently introduced a chloramphenicol resistance gene into a unique restrictionsite of this plasmid. The resulting vector, designated pLP825 (table 2) was used to transform a number of *Lactobacillus* strains.

Table 2 shows the results of electroporation experiments with representative strains of three indigenous *Lactobacillus* species of the intestinal microflora and, as a positive control, a *L.casei* strain which can be efficiently transformed following this technique by plasmid DNA (Chassy and Flickinger, 1987). *L.acidophilus, L.fermentum, L.brevis* strains are transformed with pLP825 with about equal efficiency. Compared to the control strain, the efficiency of transformation is at least three orders of magnitude lower. Plasmid DNA analysis revealed that all transformants harboured an extra plasmid indistinguishable from pLP825 used originally for transformation (not shown).

Serum antibody titers

After immunization with *Lactobacillus*-TNP only in the i.p. primed animals a specific antibody response (IgM) was observed (figure 1). Nevertheless we recently

Table 2
Transformation of *Lactobacillus* with plp825[1] by electroporation.

Strain[2]	Transformation efficiency[3]
L.casei ATCC 393	10^6-10^7
L.acidophilus NCK89	10^2-10^3
L.fermentum NCK127	10^2
L.brevis VK3	10^2

(1) The *E.coli-Lactobacillus* shuttle vector pLP825 consists of pBR322, the *cml* gene of the *Staphylococcus aureus* plasmid pC194 and a 1.8 kb cryptic plasmid from *L.plantarum* ATCC 8014 (Posno et al., 1988).
(2) The origin of *L.acidophilus* NCK89 and *L.fermentum* NCK127 has been described before (Muriana and Klaenhammer, 1987; Barefoot and Klaenhammer, 1983). *L.brevis* VK3 was kindly provided by Dr. R. Havenaar (CIVO-TNO, Zeist, The Netherlands).
(3) Expressed as the number of colony forming units per μg DNA.

showed that oral priming resulted in specific anti-TNP responses as determined by detection of specific antibody forming cells in tissue sections (Gerritse et al., 1991a). No specific anti-TNP and anti-*Lactobacillus* serum antibodies of the IgA isotype could be detected after both oral administration and i.p. booster (results not shown).

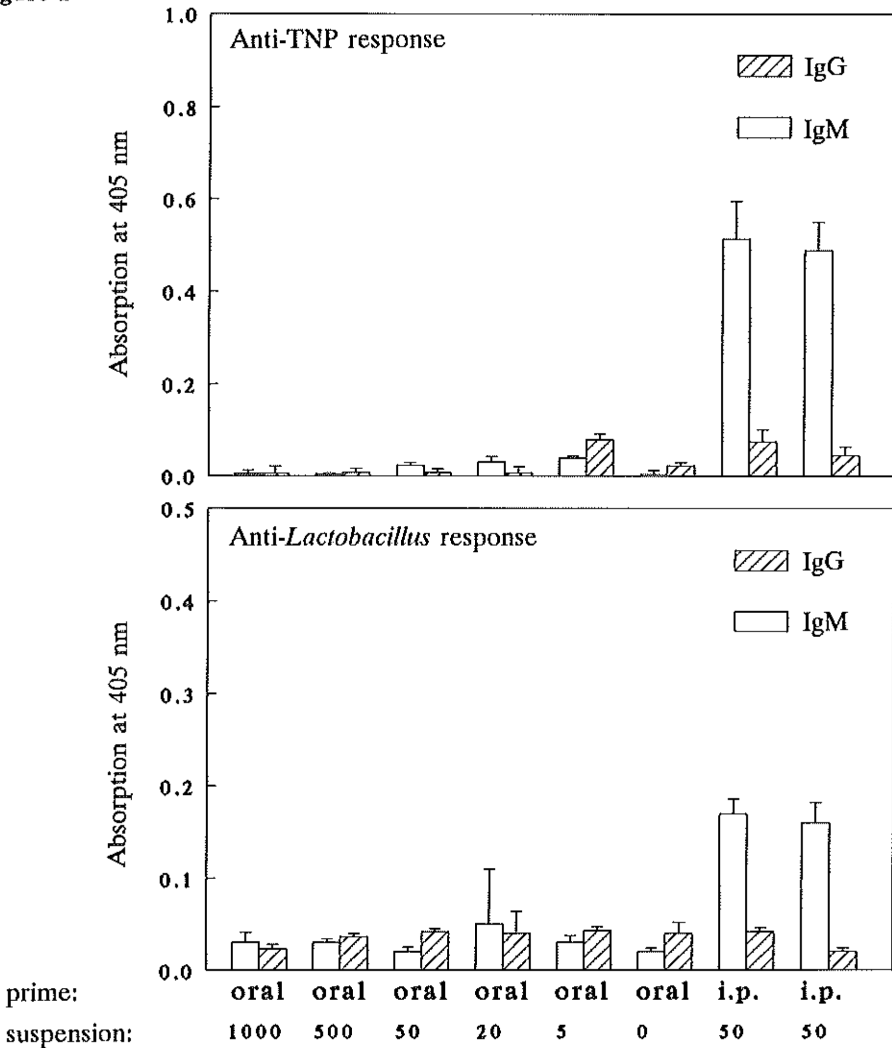
Oral administration and subsequent i.p. booster injection resulted in a specific anti-TNP as well as a specific anti-*Lactobacillus* response, except for those animals which were immunized with the suspension with the lowest TNP/*Lactobacillus* ratio or with the non-conjugated bacteria suspension (figure 2).

The majority of the antibodies specific for TNP and *Lactobacillus* are of the IgG isotype. The specific IgG and IgM responses of the orally primed and i.p. boosted animals (suspension 50) are of similar strenght as compared to the specific responses of the mice immunized twice i.p. with the same conjugate. In this experiment it is shown that the specific anti-TNP response is dependent on the *Lactobacillus*-TNP ratio of the suspension used for oral administration. The anti-*Lactobacillus* responses, in all boosted mice, are of the same magnitude.

Discussion

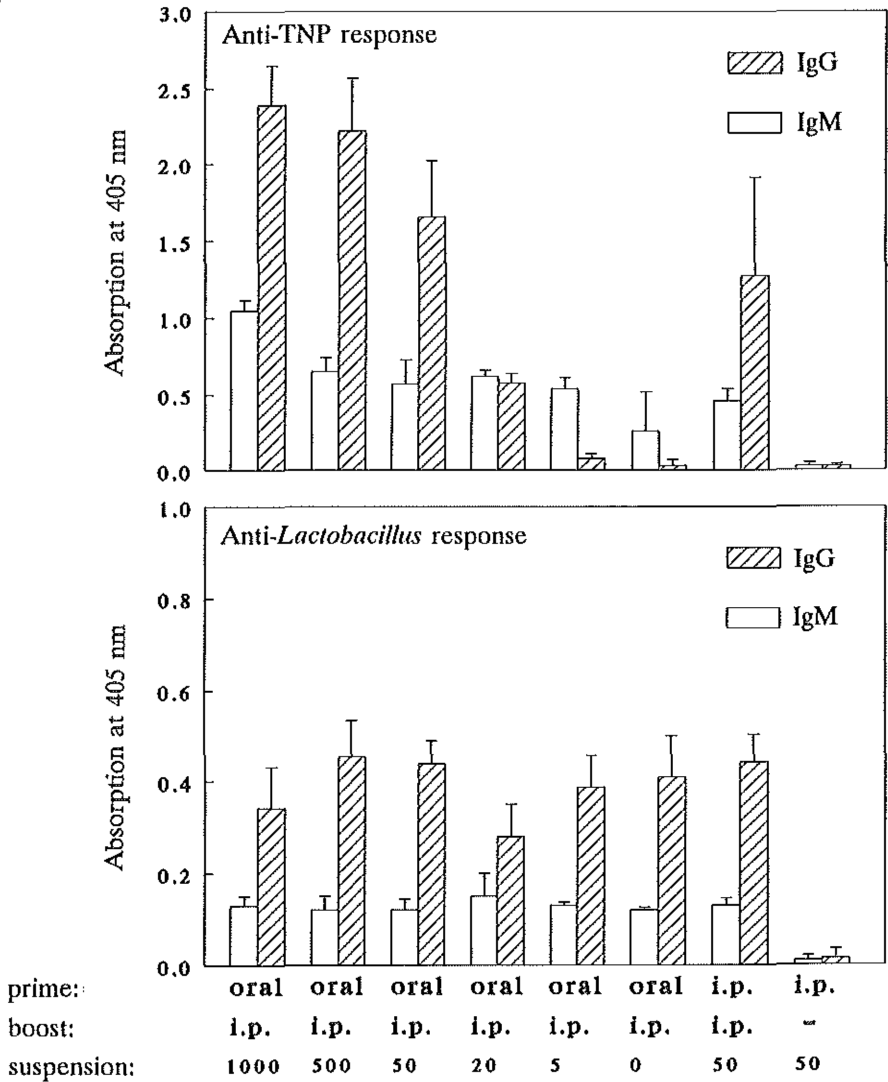
A basic requirement for the introduction of foreign antigens in Lactobacillus is the availability of a reliable transformation protocol. Our results demonstrate that different *Lactobacillus* species can be stably and reproducibly transformed with the *E.coli-Lactobacillus* shuttle-vector pLP825 by making use of the electroporation technique. Apparently, this replicon of *L.plantarum* is also functional in *Lactobacillus* species which occur in an entirely different ecological niche. The efficiency of

Figure 1



The sera of the mice were screened for specific anti-TNP and anti-*Lactobacillus* Ab's in a direct ELISA on a coating of ovalbumin-TNP and *Lactobacillus* respectively. Alkaline phosphatase labelled goat anti mouse IgM and IgG Ab's and para-nitro-phenylphosphate as substrate were used for detection. The results of 1:50 dilutions of sera collected on day seven after priming are shown.

Figure 2



The sera of the mice were screened for specific anti-TNP and anti-*Lactobacillus* Ab's in a direct ELISA on a coating of ovalbumin-TNP and *Lactobacillus* respectively. Alkaline phosphatase labelled goat anti mouse IgM and IgG Ab's and para-nitro-phenylphosphate as substrate were used for detection. The results of 1:50 dilutions of sera collected on day seven after i.p. booster injection are shown.

transformation is strain-dependent and may vary from a few transformants to up to more than 10^6 transformants per μg DNA (table 2). This implies that shotgun cloning in *Lactobacillus*, at least in some strains, has come within reach now. To date relatively little is known of (heterologous) gene in expression in *Lactobacillus*. Therefore, our current molecular biological research is directed to the generation of fundamental insights in gene expression in *Lactobacillus* and to the construction of different *Lactobacillus* expression systems for the (over)production of homologous and heterologous proteins.

The present study demonstrates that orally administered *Lactobacillus*-TNP evoke a specific anti-TNP antibody response in serum, as could be concluded from the IgG serum titers after booster injection. There was a direct relation between the anti-TNP antibody titers and the *Lactobacillus*-TNP substitution ratio of the orally administered *Lactobacillus*. However, a minimal TNP load on the bacterial membrane is essential, otherwise memory formation fails to occur, as could be revealed from the serum antibody titers of the animals which were immunized with the suspension with the lowest TNP/*Lactobacillus* ratio.

No detectable specific IgA antibodies were demonstrated in the sera both after prime and booster injection. The majority of the IgA precursor cells are derived from Peyer's patches (Craig and Cebra, 1971, Husband and Gowens, 1978). After stimulation they migrate into the circulation and develop into cells with cytoplasmic IgA (Roux et al., 1981). From the circulation these cells distribute over the mucosal tissues with a preference for the organ in which they were induced (Pierce and Cray, 1982). Restimulation of these cells by i.p imunization is not likely to occur. That IgA antibodies were not detected in the sera could also be due to the use of a heat killed Lactobacillus suspension. The underlying mechanisms which causes the differences in immune responses between replicating and non-replicating antigens upon oral administration are not yet fully understood (Wold et al., 1989). Usually non-replicating (dead) antigens, like food-antigens, do not induce a specific IgA response. This was confirmed for Lactobacilli as carriers. The presence of TNP-specific antibody producing cells was demonstrated in the spleen and mesentherial lymph nodes, the latter in limited numbers only, on day 5 and 7 after the last oral administration. However, no antibodies were found in the sera. The anti-*Lactobacillus* responses in all immunized mice were of the same magnitude since all animals were immunized with the same dose of *Lactobacillus*.

It is surprising and important to notice that oral administration of hapten-Lactobacillus conjugates led to a level of immunological memory formation which is of similar magnitude to the level of memory formation in i.p. immunized mice. Although it remains uncertain whether the immunological memory formation is due to the adjuvans capacities or the vehicle properties of *Lactobacillus*, the results of this study open new possibilities to use the non pathogenic *Lactobacillus* as a potential vaccine carrier. Experiments with transformed *Lactobacillus* are in progress.

CHAPTER 4.3

MUCOSAL IMMUNE RESPONSES AND SYSTEMIC IMMUNOLOGICAL MEMORY AFTER ORAL ADMINISTRATION OF TRINITROPHENYL *LACTOBACILLUS* CONJUGATES IN MICE

K Gerritse, M Posno*, MJ Fasbender, MM Schellekens,
WJA Boersma and E Claassen

TNO Prevention and Health, Leiden, The Netherlands
**TNO Nutrition and Food Research, Rijswijk, The Netherlands*

Lymphatic Tissues and *in vivo* Immune Responses 84:497-504, 1991

Summary

Lactobacillus can be used to deliver antigens via the oral route to the immune system to generate a humoral immune response to antigens which are associated to the bacterial membrane. This chapter describes the routing of *Lactobacillus* associated antigens and the *in situ* localization of antigen specific AFCs in lymphoid organs, after both oral administration and intraperitoneal injection of a *Lactobacillus* associated model antigen, trinitrophenyl. The data discussed here show that at least a part of the TNP-Lactobacillus, is transported directly to the mesenteric lymph nodes (MLN), as could be concluded from the presence of antigen-complexes in MLN tissue sections.

Introduction

The oral route is a very convenient route of vaccination in comparison with the more frequently used parenteral routes, especially in vaccination programs in which large numbers of subjects are involved. In addition, the activation of the mucosal immune system as well as systemic responses can be obtained by oral application of antigens. Although this sometimes results in an adequate local expression of immunoglobulin A (IgA) responses in various mucosal tissues and secretions (Mestecky, 1978), the oral administration is generally very inefficient, requiring large quantities of immunogen and yielding only modest systemic antibody responses. With regard to numerous pathogens, a local response only is not sufficient and a systemic response obligatory.

Owing to a digestive processing, many antigens loose their immunogenic potential. A suboptimal amount of antigen reaches the mucosal immune system and,

therefore, requires an adjuvant to evoke or enhance the immune response. Transformed microorganisms can be used as antigen carriers, but most of these microorganisms (e.g., *Escherichia coli, Streptococcus mutans, Salmonella typhi*; (Leclerc et al., 1989)) are generally recognized as pathogens and are not acceptable for general application. In this respect, the use of a GRAS (Generally Recognized as Safe) organism like *Lactobacillus* may be preferred.

The use of *Lactobacillus* as a vector antigen delivery system has several other potential advantages over other microorganisms besides the above mentioned GRAS status. *Lactobacillus*, in mammals, is able to pass the digestive tract and colonize in the gut. In addition, adjuvant properties of *Lactobacillus* have been described (Perdigón et al., 1986c). As for many other microorganisms, it is possible to produce transformations of *Lactobacillus* with foreign deoxyribonucleic acid (DNA). Strains of *Lactobacillus* which produce a foreign antigen epitope, which is expressed on the membrane surface or is secreted intra- or extracellularly, can be constructed.

Considering the findings mentioned above, we recently initiated investigations on: (a) development of gene-transfer and gene-expression systems for the introduction and efficient expression of antigens in *Lactobacillus* species which are (natural) inhabitants of the gastrointestinal tract, and (b) immunological evaluation and optimization of the immune responses (mucosal and systemic) that are generated through presentation of *Lactobacillus*-associated antigens by the oral route.

The aim of the work presented here was a comparative study on the effects of *Lactobacillus* administered by the oral or peritoneal route on the generation of the systemic response. To evaluate this a small hapten, trinitrophenyl (TNP), coupled to *Lactobacillus* was used for oral immunization purposes, serum titers were determined, and lymphoid tissues were screened for specific antibody forming cells (AFCs).

Although life antigen carriers are more immunogenic at mucosal surfaces than nonreplicating antigen carriers (Lycke and Holmgren, 1986), most live antigen carriers have an undesirable potentiation of an immune response to a large number of bystander antigens found in the gastrointestinal tract. For this reason, the *Lactobacillus* microorganisms were heat killed prior to trinitrophenylization. Furthermore, Bloksma et al. (1979) revealed that viable *Lactobacillus* microorganisms stimulate exclusively delayed hypersensitivity, whereas heat-killed bacteria had an adjuvant effect on antibody formation. The potential use of *Lactobacillus* as a antigen carrier system will be discussed.

Material and methods

Cultivation of *Lactobacillus*

Lactobacillus acidophilus (strain NCK89) was grown on Man Rogosa Sharp (MRS) broth (Difco, Detroit, Michigan, USA). After aerobic incubation at 37°C

overnight, the organisms were harvested by centrifugation at 3640 g for 15 min at room temperature and resuspended in phosphate-buffered saline (PBS) ($1x10^{10}$ $CFU.ml^{-1}$). Subsequently the suspension was heated for 30 min at 60°C.

Conjugation

In order to obtain TNP-*Lactobacillus* conjugate, 50 μl of a 2,4,6-trinitrophenyl sulphonic acid (TNBS) solution (20 $mg.ml^{-1}$ PBS) was added to 1 ml *Lactobacillus* suspension (53 $mg.ml^{-1}$ PBS). The suspension was incubated for 1 hr at room temperature in the dark, washed, and resuspended in 1 ml PBS. The TNP-alkaline phosphatase (TNP-AP) conjugates were obtained essentially according to the method of Claassen and Van Rooijen (1984).

Trinitrophenylated ovalbumin was used for the detection of anti-TNP antibodies in a direct enzyme-linked immunosorbent assay (ELISA). The TNBS (100 mg) was added under gentle stirring to 100 mg ovalbumin in 10 ml borate buffer (0.1 M, pH 9). The solution was incubated in the dark for 2 hr at room temperature, and dialyzed against PBS.

Immunization

Lactobacillus-TNP conjugates were orally administered three times on alternating days to groups of female mice (Balb/c 12-15 weeks old). Each oral administration contained 500 μg *Lactobacillus* suspended in 500 μl 0.2 M $NaHCO_3$ to neutralize hydrochloric acid in the stomach and reduce degradation of the antigen determinants to a minimum (Black et al., 1983). Four weeks after the last administration, the mice were boosted orally three times on alternating days. Control groups were both i.p. primed and boosted with 500 μg TNP-*Lactobacillus* conjugates suspended in 250 μl PBS. The mice were sacrificed 3, 5, 7 and 21 days after the last oral administration or i.p. prime injection and five day's after the last booster administration or booster injection. Blood samples of the mice were collected by heart puncture. The sera were screened for anti-TNP and anti-*Lactobacillus* antibody (Ab) reactivity. The spleen (SP) and mesenteric lymph nodes (MLNs) were removed and stored in liquid nitrogen. Tissue sections of the organs were screened for anti-TNP specific AFCs and antigen trapping.

Direct ELISA

Polyvinyl chloride (PVC) microtiter plates (Titertek, Flow Laboratories, Irvine, Scotland) were coated with TNP-ovalbumin (5 $\mu g.ml^{-1}$ PBS, 50 μl/well). To block nonspecific binding a second coating of gelatine (5 $mg.ml^{-1}$, 50 μl/well) was applied. The plates were incubated for 1 hr at 25°C with dilutions of TNP-induced antisera and preimmunization sera to correct the TNP-induced antisera for background reactivity. Sera dilutions were added to three separate plates. The plates were washed and separately incubated with either alkaline phosphatase-labelled goat-antimouse IgA, IgM or IgG (H + L) (KPL, Inc., Gaithersburg, Maryland, USA)

1:2000 in PBS. After washing 50 μl para-nitrophenylphosphate (Boehringer, Mannheim, West Germany) solution (1 $mg.ml^{-1}$) in 10 mM diethanolamine (1 mM $MgCl_2$, pH 9.8) was added to each well. After 30 min, the absorption at 405 nm was determined.

The anti-*Lactobacillus* serum titer was determined on glutaraldehyde-preactivated polystyrene microtiterplates (NUNC, GIBCO Europe, Breda, The Netherlands). The ELISA was performed as was indicated for the TNP-ovalbumin coated microtiter plates.

Tissue preparation and immunocytochemical staining

Mice were sacrificed by CO_2 euthanasia, SP and MLNs were removed and snap frozen in liquid nitrogen. Eight micrometer cryostat tissue sections were fixed for 10 min in acetone containing 0.02 % (v/v) hydrogen peroxidase. For determination of the antigen specificity of the AFCs in MLNs or SP, tissue sections were incubated overnight with TNP-AP dilutions in 0.1 % bovine sera albumin (BSA) in PBS (w/v). The AP activity was revealed according to the method of Burstone (1958) with some modifications (Claassen et al., 1986d).

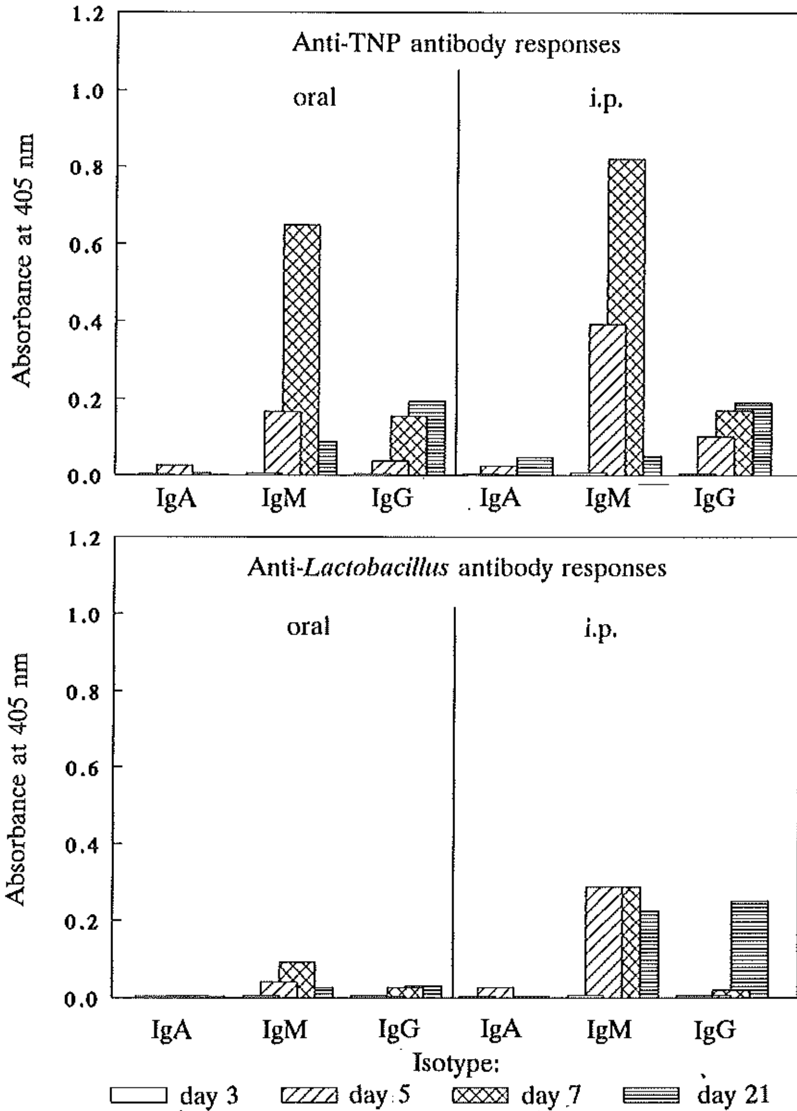
Trapped *Lactobacillus* conjugate was detected in a two-step staining procedure. Slides were incubated overnight at 4°C with purified rabbit anti-*Lactobacillus* Abs (2 $mg.ml^{-1)}$ diluted 1/15 to 1/50 in a solution of BSA (1 $mg.ml^{-1}$ PBS). After washing, the slides were incubated for 60 min at RT with goat-antirabbit IgG-horseradish peroxidase (HRP) conjugate diluted 1/100 in a solution of BSA (1 $mg.ml^{-1}$ PBS) and normal mice sera (1% v/v). The HRP activity was revealed as described previously (Claassen et al., 1986).

Results

ELISA analysis of the sera antibody titers

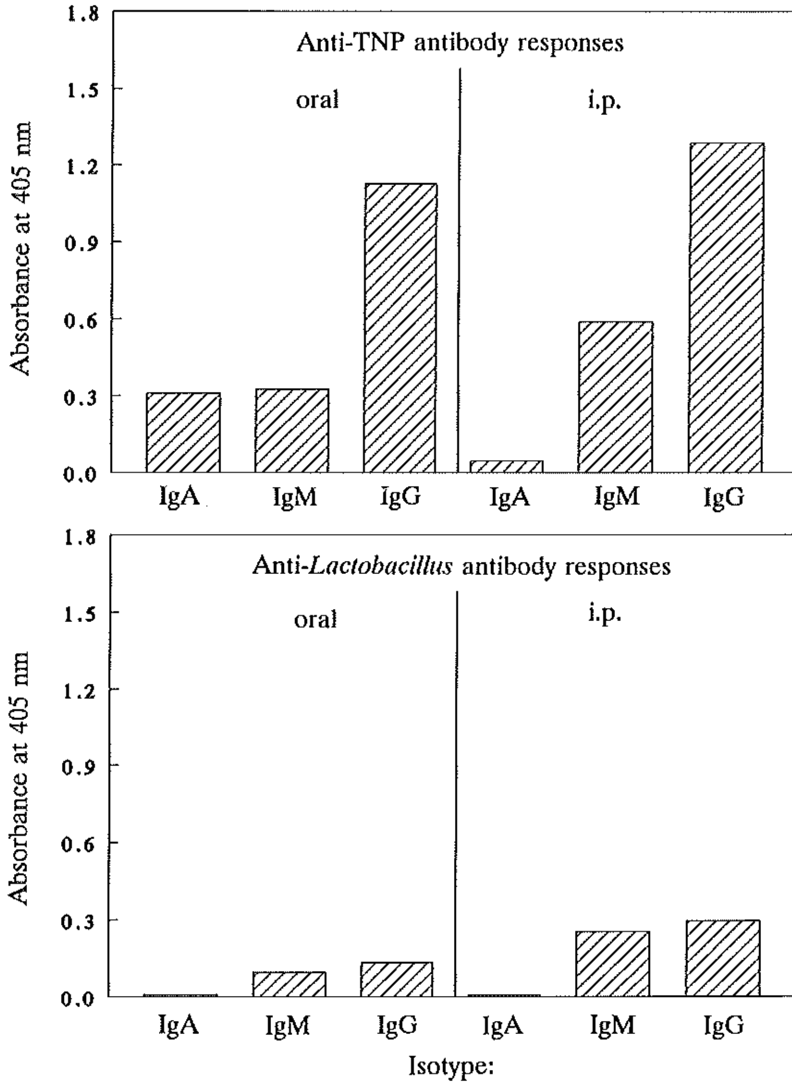
In both oral- and intraperitoneal primed animals, a specific anti-TNP antibody response (IgM) was observed with a peak level on day 7 after prime. Only in the intraperitoneal primed mice, a low IgM anti-*Lactobacillus* response was detected (figure 1). No specific anti-TNP and anti-*Lactobacillus* serum antibodies of the IgA or IgG isotype could be detected after both oral or i.p. prime. In both groups, the booster administration or injection resulted in a specific anti-TNP as well as a low specific anti-*Lactobacillus* response (figure 2). Immunoglobulin G is the prevailing isotype of the anti-TNP and anti-*Lactobacillus* antibodies after boost in both groups. In the oral boosted animals, also a significant anti-TNP IgA response was detected. The anti-TNP IgG response of the orally primed and boosted mice is of similar strength as compared to the specific anti-TNP IgG response of the animals immunized twice i.p.

Figure 1



Sera of the mice were screened for specific anti-TNP and anti-*Lactobacillus* Abs in a direct ELISA on a coating of ovalbumin-TNP and *Lactobacillus*, respectively. Alkaline phosphatase-labelled goat-antimouse IgA, IgM and IgG Abs and para-nitrophenylphosphate as substrate were used for detection. The results are shown of 1:50 dilutions of sera collected on days 3, 5, 7, and 21 after prime.

Figure 2



Analysis of serum anti-TNP and anti-*Lactobacillus* antibodies in ELISA. The assay conditions are as indicated for figure 1. The results are shown of 1:50 dilutions of sera collected on day 5 after booster injection or last booster administration.

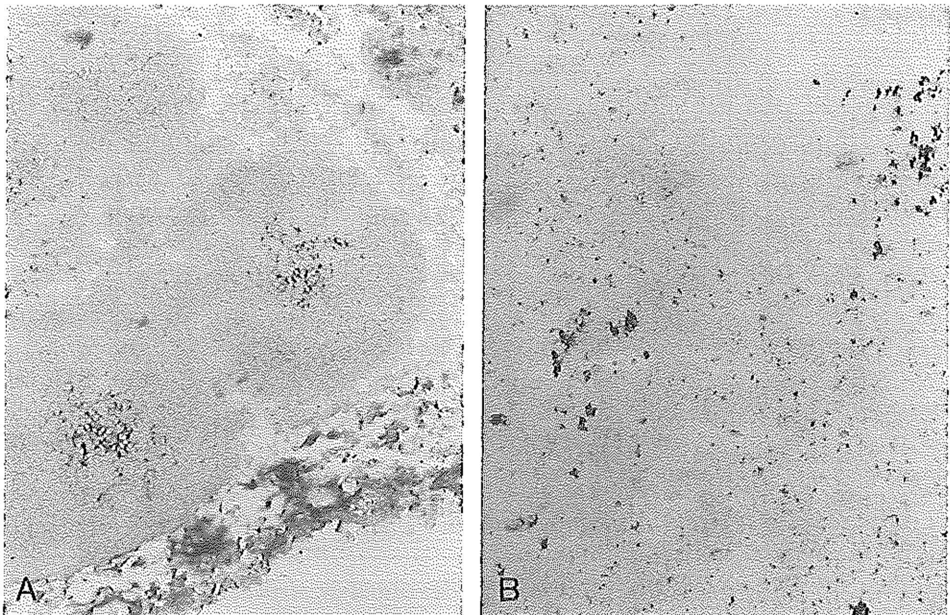


Figure 3
MLNs and SP of the animals, fed with a suspension of TNP-*Lactobacillus* conjugate, were removed on day 5 after the last oral prime administration. Tissue sections of mesentherial lymph nodes (a) and spleen (b) were stained with TNP-AP conjugate.

Table 1

Time: (days)	Immunization: Tissue:	oral MLN	i.p. MLN	oral SP	i.p. SP
3		0	0	3.4	1.7
5		1.4	0	7.9	6.2
7		1.2	0	3.1	5.1
21		0.4	0	2.0	2.1

Number of specific anti-TNP antibody-forming cells in MLNs and SP tissue sections after oral or i.p. priming. The number of AFCs was calculated per square mm^2 on day 3, 5, 7 and 21 after immunization. Cells were detected by TNP-AP staining.

Immunocytochemical Detection of TNP-Specific AFCs

In the spleens of both the oral- and intraperitoneal-immunized mice, specific anti-TNP antibody-forming cells were detected on all sample day's (figure 3). The number of AFCs in the spleen sections is of the same magnitude in both groups (table 1). Specific AFCs in the MLNs are only observed in the oral-primed animals (figure 3). The greatest number of AFCs were found in the tissue sections of the MLNs removed on day 5 after the last oral administration. Antigen trapping in immune complexes was observed in MLN tissue sections of the oral immunized animals by staining with TNP-AP (see figure 3). The antigen trapping was verified by staining alternating tissue sections with anti-*Lactobacillus* antibodies (results not shown). The immune complexes were detected in the lymphoid follicles; this observation is in accordance with the normal localization of immune complexes as reviewed by Van Rooijen et al. (1989).

Discussion

In a previous study we demonstrated that orally administered TNP-*Lactobacillus* conjugates evoke a specific anti-TNP IgG antibody response in serum after an i.p. booster injection (Gerritse et al., 1990b). We now demonstrate that oral priming followed by an oral booster administration also results in a specific anti-TNP response, as could be revealed from the serum IgG antibody titers. This response was of the same magnitude as the antibody response in serum of the animals immunized twice i.p. This implies that an anti-TNP response is equally effective elicited by oral administration of TNP-*Lactobacillus* conjugates as by i.p.immunization. In addition, the enumeration of the anti-TNP AFCs in tissue sections of the spleen led to similar results.

To get similar levels of serum antibody responses, it is necessarily to administrate orally three times the *Lactobacillus* conjugate. Oral administration of the TNP-*Lactobacillus* conjugate only once or twice is not sufficient to evoke a specific response (results not shown). This could be explained in two ways. One possibility is that only a small part of the total amount of TNP-*Lactobacillus* reaches the mucosal epithelium and is only partly internalized by either the epithelium cells or the specialized M cells, whereas the greater part passes the digestive tract. Another possibility is that when antigens enter the mucosa, they mainly stimulate IgA precursor lymphocytes owing to the fact that the greatest population of B cells in the GALT are IgA lymphocytes. For this reason, it is not surprising that there is a larger amount of antigen needed to stimulate the relatively small population of non-IgA lymphocytes.

Only in the sera of the orally boosted mice was a specific IgA response observed. In previous experiments, no IgA response was observed after oral priming subsequently followed by an i.p. booster injection, suggesting that IgA-producing lymphocytes can only be restimulated via the oral route.

The occurrence of an IgA response could be explained in two ways. The majority of the IgA precursor cells are derived from Peyer's patches (Craig et al., 1971). After these cells are stimulated for the first time they migrate via the MLNs and thoracic duct into the circulation. Finally, they return to the mucosal tissues with a preference for the organ they were initially stimulated by (Pierce et al., 1982). After restimulation, the greater part of the Abs produced will be excreted as secretory IgA in the intestinal lumen, whereas a small amount will be released as monomeric IgA to the circulation. Alternatively, it is possible that a part of the precursor IgA cells do not return to the mucosal lymphoid tissues after the first stimulation but remain in the MLNs. Since trapped TNP-*Lactobacillus* could be detected in the MLNs after oral immunization, restimulation might occur. Determination of the isotype of the specific AFCs in the MLN which occur during the time coarse between prime and boost could help to clarify this issue.

In both groups, low IgM and IgG and no specific IgA anti-*Lactobacillus* responses were measured after boost. This observation confirms the low intrinsic antigen properties reported by other investigators (Carlsson and Bratthall, 1985). The low responses could be due to the use of a heat-killed *Lactobacillus* as carrier. Usually, life antigens induce better immune responses than nonreplicating antigens (Fuhrman and Cebra, 1981).

When antigens are internalized in the mucosal tissue it is believed that antigen is processed by antigen-presenting cells and subsequently presented to T lymphocytes or B lymphocytes in the lamina propria or Peyer's patches, respectively. In this study, it has been shown that at least a part of the TNP-*Lactobacillus*, whether or not processed, is transported directly to the MLNs, as could be concluded by the presence of antigen complexes after staining with TNP-AP and anti-*Lactobacillus* in alternating MLN tissue sections.

The low intrinsic antigenic properties of the *Lactobacillus* together with the observation that oral prime and booster administrations led to a comparable specific antibody response obtained after i.p. immunization suggests the possible usage of the *Lactobacillus* as an antigen carrier for oral immunization purposes.

CHAPTER 4.4

LACTOBACILLUS AS A VECTOR FOR ORAL DELIVERY OF ANTIGENS: THE ROLE OF INTRINSIC ADJUVANTICITY IN MODULATION OF IMMUNE RESPONSES

K Gerritse, K Maassen, R Leer*, D Heemskerk,
WJA Boersma and E Claassen

TNO Prevention and Health, Leiden, The Netherlands
**TNO Nutrition and Food Research, Rijswijk, The Netherlands*

Submitted for publication

Summary

The influence of the adjuvant activity of *Lactobacillus* strains on the activation or tolerization of the immune system was investigated. *Lactobacillus plantarum 80*, transformed to produce *Escherichia coli* ß-galactosidase, were orally administered to BALB/c mice, prior to intraperitoneal challenge with purified ß-galactosidase. In animals fed with ß-galactosidase producing *Lactobacillus plantarum 80* a suppression (up to 64%) of the anti ß-galactosidase serum responses was observed after subsequent antigen injection. The data presented in this study show that oral administration of an antigen by *Lactobacillus plantarum 80*, a strain with relatively low adjuvant properties can lead to the induction of a state of peripheral tolerance.

The influence of oral administration of a *Lactobacillus* strain, with relative high adjuvant activity, mixed with antigen, on an immune response induction with development experimental autoimmune encephalomyelitis (EAE) in mice as a read out system was also investigated. A suspension of *Lactobacillus casei ATCC393* and antigen was administered orally, prior to disease induction with the same antigen. The simultaneous oral administration of *Lactobacillus casei ATCC393* and antigen resulted in a significant enhancement of the immune response.

The results of this study indicate that dependent on strain specific adjuvanticity *Lactobacillus* are potential candidates for the oral delivery of immune potentiating signals as well as for the induction of peripheral immunological non-responsiveness against putative autoantigens in human autoimmune diseases.

Introduction

Oral administration of antigens may lead to a systemic antigen specific IgG antibody response, without a significant IgA response (Gerritse et al., 1990b; 1991; Peri et al., 1982). However, dependent on specific conditions, oral administration of antigens can either induce a relative state of specific immunological non-responsiveness to a subsequent parental challenge with the same antigen (Vaz et al., 1977; Miller and Hanson, 1979; Thompson and Staines, 1990; Brod et al., 1991) or may lead to the production of antigen specific secretory IgA antibodies at the intestinal mucosa and other distant mucosal sites (i.e. respiratory and genital tract).

It is generally assumed that the presentation and processing of tolerance inducing and immunogenic proteins are the same. This suggests that there are certain additional requirements/conditions which determine, whether the immune system will be stimulated or will reach a certain state of tolerance. Experiments performed by Melamed and Friedman (1993) showed that a single intra-gastric delivery of ovalbumin induced a transient form of tolerance. Pulsed oral administration of ovalbumin, one single administration per day, for 14 days was not able to induce tolerance in mice. CBA and SWR/J mice, aged 2-3 months, were only tolerized, when antigen was administered semi-continuous, i.e. throughout the day by voluntary ingestion (Stokes et al., 1983). In addition, it was demonstrated that the semi-continuous oral administration of small amounts of antigen is an effective condition for the induction of tolerance (Saklayen et al., 1984; Heppell and Kilshaw, 1982). Genetically modified microorganisms can be used for the continuous production of antigens. Genetically modified microorganisms which colonize the gastrointestinal tract after a single or perhaps a few oral administrations, may effectively fulfil the requirements for an oral tolerance inducing delivery system.

It has been shown that *Lactobacillus* can maintain a stable population in both animal and human gut (Savage, 1977; Pouwels and Leer, 1993; Chassy, 1987; Perdigon et al., 1988) and lactobacilli have mucosal adhesive properties (Lencner et al., 1987). In previous studies we have shown oral administered *Lactobacillus* to be very efficient as antigen presenting vehicles (Gerritse et al., 1990b; 1991a). Furthermore, gene transfer and expression systems for an efficient production of foreign antigens by a variety of *Lactobacillus* species have been developed recently (Posno et al., 1991a; Pouwels et al., 1992). In addition, *Lactobacillus* exhibit a low intrinsic immunogenicity as compared to other microorganisms (Carlsson and Bratthall, 1985) and the presence of *Lactobacillus* in the gastro-intestinal tract can inhibit outgrowth of microorganisms causing infectious diseases (Perdigon et al., 1986c; 1988). Taking together, these data indicate that *Lactobacillus* might be a convenient microorganism to be used as antigen carrier and producer simultaneously, for delivery of antigens to the immune system by the oral route.

In this paper we describe two types of experiments to study the effects of oral administration of antigens on the immune system. For this purpose we used two

Lactobacillus strains, which differ in their adjuvant properties. First, we have studied the effects on the immune system of oral antigen delivery and presentation using a *Lactobacillus* strain with relatively low adjuvant properties, in order to induce immunological tolerance. To this end, low levels of ß-galactosidase producing *Lactobacillus plantarum 80* (LP80), were administered orally to mice. Serum anti-ß-galactosidase antibody responses were determined after intraperitoneal challenge injections with ß-galactosidase. Subsequently, immune reactive or tolerant spleen cells were transferred to naive recipient mice and serum anti-ß-galactosidase antibody responses were determined after ß-galactosidase booster injection. The effects oral administration of antigen and a *Lactobacillus* strain with high adjuvant properties, *Lactobacillus casei* (*L.casei*), was studied in mice using an immune disease induction model. To this end EAE was induced in mice with an encephalitogenic proteolipid protein (PLP) peptide. Induction with PLP-epitope residues 139-151 results in the development of acute EAE, which is clinically and pathologically identical to EAE induced by sensitization with whole CNS myelin or with MBP (Tuohy et al., 1989; Sobel et al., 1990). A suspension of *L.casei*, and the synthetically produced PLP T-cell epitope (Tuohy et al., 1989) were simultaneously administered orally to mice. Subsequently, EAE was induced by immunization with PLP-peptide in adjuvant. The effects of the simultaneous oral delivery of the PLP-peptide and high adjuvant strain *L.casei* on the course of disease, were monitored by evaluation of clinical characteristics.

Material and methods

Construction of LP80 producing *E.coli* ß-galactosidase

The *Lactobacillus* expression vector pLPCR2 (Forrest, 1988) was obtained by inserting the promoter of the *xylR* gene and the terminator of the *xylB* gene of *Lactobacillus pentosus* (separated by a multiple cloning site) in the *Escherichia coli* - *Lactobacillus* shuttle-vector pLP3537 (Carlsson and Bratthall, 1988; Forrest, 1988). To construct pCBH72, the promoter of the *xylR* gene in pLPCR2 (Pouwels et al., 1992) was replaced by a fragment comprising the promoter of the conjugated bile acid hydrolase gene of *Lactobacillus plantarum 80* (LP80) (Christiaens et al., 1992) and the *E.coli lacZ* gene was inserted into the multicloning site.

LP80 was cultivated and transformed by electroporation as described previously (Christiaens et al., 1992). LP80 transformants were selected on solid MRS medium supplemented with 10 μg/ml erythromycin. ß-Galactosidase production was confirmed in the lysate of LP80 transformants by standard gel electrophoresis, immunological techniques and protein staining. ß-Galactosidase producing and non ß-galactosidase producing LP are designated LP80$^+$ and LP80$^-$ respectively.

Antigen administration by LP80⁺ and immunization

Six groups of female BALB/c mice (10-12 weeks) received orally 0.05 (I), 0.1 (II), 0.5 (III) and 1 (IV) mg dry weight $LP80^+$, 1 mg dry weight $LP80^-$ (V) or PBS (VI) only. The ß-galactosidase expression levels in $LP80^+$ were approximately 1-2% of total protein contents. The estimated gastrointestinal passage time is about two days. To mimic a continuous delivery of antigens during a period of ten days, *Lactobacillus* administrations (500 μl) were repeated five times with a two day interval. At days 3 and 31 after the last oral administration all mice were boosted (booster injection I and II) i.p. with 8 μg ß-galactosidase in 200 μl PBS (table 1). The amount of antigen (8 μg) used for booster injection was a low dose of ß-galactosidase which is just sufficient to mount an anti-ß-galactosidase serum Ig response after an i.p. booster injection in low dose ß-galactosidase primed mice (data not shown). Serum anti-ß-galactosidase responses were determined in a direct ELISA.

Transfer of spleen cells

Mice were sacrificed by CO_2 euthanasia and the spleens were removed. Erythrocytes were removed by standard ammoniumchloride treatment (Mishell et al., 1980). Cells of individual spleens (500 μl) were injected i.v. in naive, 5 Gy irradiated, recipient female BALB/c mice (10-12 weeks old). Two days after cell transfer mice were boosted (III) by i.p. injection with 8 μg β-galactosidase in 200 μl PBS (table 1). The sera were screened for anti-β-galactosidase antibody reactivity in a direct ELISA.

ELISA

PVC microtiter plates were coated with β-galactosidase (5 $\mu g.ml^{-1}$, 50 μl/well) overnight at 4°C. Subsequently free sites were blocked for 30 min at 25°C with gelatin (0.5 $mg.ml^{-1}$ PBS, 50 μl/well) to prevent non-specific antibody binding. After washing (5x) with PBS-G (0.1 mg gelatine/ml PBS) the plates were incubated for 60 min at 25°C with dilutions of immune sera. The plates were washed (5x) with PBS-G and incubated with alkaline-phosphatase labelled goat anti-mouse Igtotal (i.e. IgG, IgM, IgA)(KPL, Inc., Gaithersburg, USA) for 60 min at 25°C. The plates were washed (5x) with PBS-G and incubated with paranitrophenyl phosphate (1 $mg.ml^{-1}$ 10 mM dietanolamine,1 mM $MgCl_2$, pH 9.8) as enzyme substrate. After incubation for 30 min at 25°C the absorption was determined at 405 ηm.

Peptide synthesis, purification and characterization

The synthesis of the PLP-peptide was carried out with 9-fluororenylmethoxycarbonyl (f-moc) protected aminoacids, following the solid phase synthesis method (Merrifield, 1963) on an automated Milligen 9050 Continuous Flow Synthesizer (Millipore Co., Bedford, MA, USA). The PLP-peptide is a 13-mer aminoacid sequence analogue to residue numbers 139-151 of rat PLP (Dautigny et

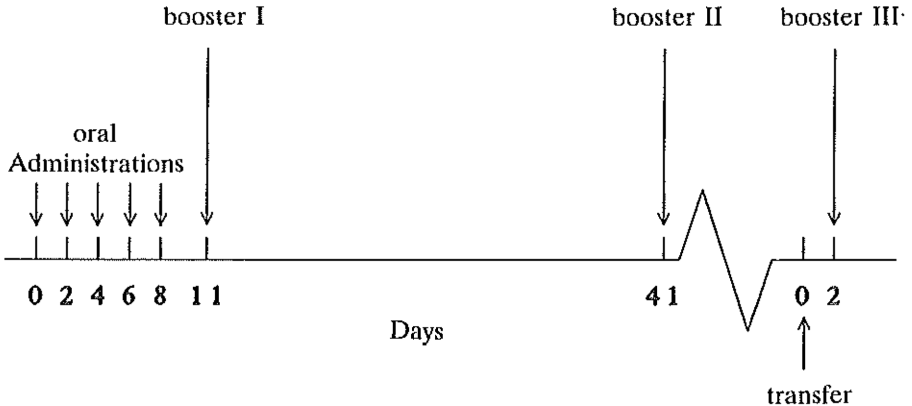


Table 1
Administration and immunization time schedule.
Different doses of β-galactosidase producing transformed *Lactobacillus plantarum 80* ($LP80^+$) were administered to four groups of mice (A: 0.05 mg; B: 0.1 mg; C: 0.5 mg; D: 1 mg). Non-transformed *Lactobacillus plantarum 80* ($LP80^-$)(E: 1 mg) and PBS (F) only were administered to control groups. Subsequently mice were boosted twice (I, II) i.p. with β-galactosidase. Individual spleen cell suspensions were transferred to naive recipient mice. Recipient mice were boosted (III) with β-galactosidase two days after transfer.

al., 1985) sequence: NH_2-His-Cys-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys-Phe-COOH. Side-chains were protected: aspargine: 2,4,6-trimethoxybenzyl; histidine and cysteine: trityl; lysine: tertiar-butoxycarbonyl. Peptide cleavage and side-chain deprotection of the completed peptide were performed by trifluoracid treatment as presented earlier (Van Denderen et al., 1987). Peptide purification was performed according to the method as was described by Shively (1986). Aminoacid composition of the synthetic peptide was confirmed by HPLC analysis according to the method of Janssen et al. (1986).

Oral administration of PLP-peptide and *Lactobacillus casei ATCC393*

Female SJL/j mice (12-15 weeks old, 8 mice per group) were pre-treated by oral administration of *L.casei* and PLP-peptide (group E and F), PLP peptide (group B and C), *L.casei* (group D) or PBS only (group A). To mimic a continuous delivery of antigens during a period of ten days, administration of suspensions or solutions (500 μl per administration) were repeated five times with a two day interval. PLP-peptide was administered in two amounts: 100 μg (group B and E) and

500 μg (group C and F). *L.casei* suspensions, administered to mice of group D, E and F, contained 5.10^8 colony forming units. EAE induction was performed three days after the last oral administration.

EAE induction

EAE induction was performed by subcutaneous injection at two sites in the abdominal flanks, with 150 μg PLP-peptide emulsified in Freunds complete adjuvant (FCA)(100 μl/injection). The emulsion contained per 100 μl: 25 μg *Mycobacteria tuberculosis* (H37RA, Difco) in 50 μl FCA and 75 μg peptide in 50 μl PBS. In addition each mouse was injected intravenously, on day 0 and 2, with 200μl *Bordetella pertussis* suspension (10.10^{10} in 1 ml PBS). The severity of EAE clinical signs was evaluated each day according to following criteria: Disability scale (DAS) grade 0 = no clinical signs, grade 1 = tail weakness, grade 2 = mild paraparesis and ataxia of the hind legs, grade 3 = severe paraparesis or ataxia of the hind legs, grade 4 = moribund, grade 5 = dead due to EAE.

Results

Oral antigen delivery

A significant reduction in the anti-β-galactosidase sera responses was measured after the second booster injection in all groups of mice pre-treated with β-galactosidase producing Lactobacilli (figure 1). After booster injection I (for administration and immunization schedule see table 1) low anti-β-galactosidase Ig-total serum responses were observed on all sample days (absorbance < 0.1 at 1/200 serum dilutions). The anti-β-galactosidase serum responses of the mice pre-treated with β-galactosidase producing Lactobacilli (groups I, II, III, and IV) did not differ significantly from the responses of the control groups (V and VI) (data not shown). After booster injection II the highest anti-β-galactosidase serum antibody responses were measured on day 8 (figure 1). The anti-β-galactosidase antibody responses (1/800 serum dilutions), of the animals which received 50, 100, 500 and 1000 μg transformed $LP80^+$ orally, were reduced with 64%, 51%, 27% and 39% respectively as compared to the anti-β-galactosidase response measured in control animals.

Post-transfer anti-β-galactosidase serum antibody responses

A reduction of the anti-β-galactosidase responses was observed after a subsequent booster injection (III) with β-galactosidase in three out of four test groups which received spleen cells of donor mice pre-treated with oral administrations of $LP80^+$. The anti-β-galactosidase responses were most reduced in sera from mice, which received the spleen cells of donor mice orally pre-treated with a low dose of β-galactosidase producing $LP80^+$ (0.1 mg)(figure 2). The highest anti-β-galactosidase responses were observed in mice which received spleen cells of 0.5 mg β-galactosidase producing $LP80^+$ pre-treated donor mice. These latter animals

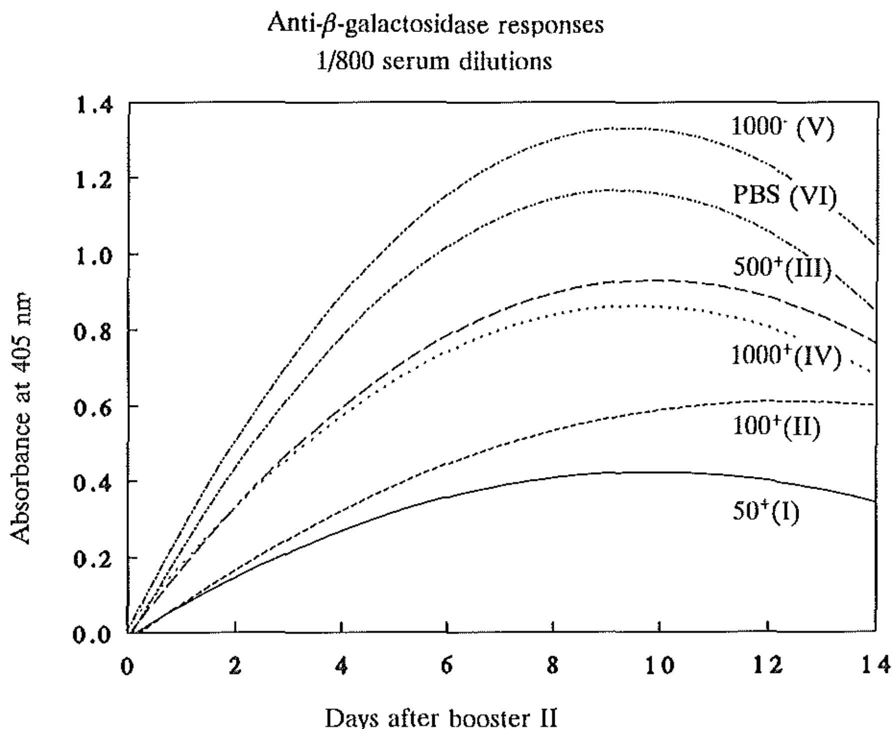


Figure 1
The anti β-galactosidase serum antibody responses on day 0, 5, 8 and 14 after β-galactosidase booster II injection (table 1) were measured in a direct ELISA (see: Material and Methods). The results are expressed as the average absorbance at 405 nm of serum dilutions (1/800) of three mice per group. The animals of the four test groups orally received 50(I), 100(II), 500(III) and 1000 μg LP80⁺(IV) *Lactobacillus plantarum* respectively, prior to booster β-galactosidase injections. The animals of the control groups received non transformed *Lactobacillus plantarum* (neg., V) or PBS (IV) only prior to booster β-galactosidase injections.

showed the lowest reduction (27%) in anti-β-galactosidase antibody responses after booster injection II. In contrast to the other three groups, the anti-β-galactosidase responses of this group of donor mice were not reduced as compared to the responses of both control groups of mice.

Role of adjuvanticity of *Lactobacillus casei* on EAE induction

Oral administration of *Lactobacillus casei* only, prior to EAE induction (group D), resulted in EAE enhancement, as compared to the course of disease of animals which were not pre-treated with *Lactobacillus casei* (A, B and C) (administration and EAE induction schedule; see table 2).

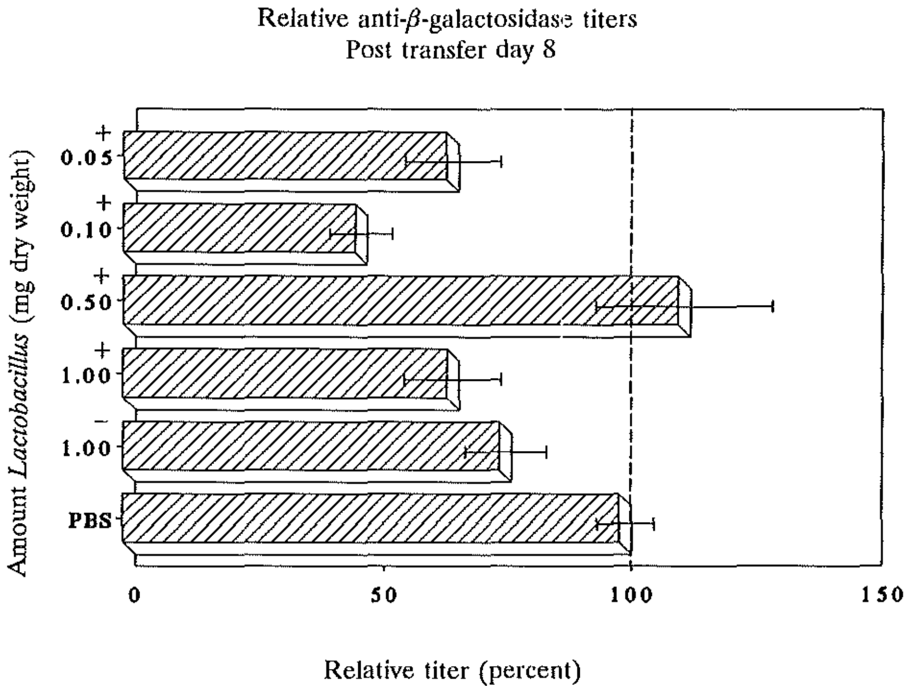


Figure 2
The anti β-galactosidase serum antibody responses on day 8 after post transfer β-galactosidase booster injection (III) (table 1) were measured in a direct ELISA (see: Material and Methods). The results are expressed as the average relative titers. The average titer of the control recipient animals which received the spleen cells of the PBS pre-treated donor animals was set at 100%. Standard deviations of the average relative titers are indicated.

Animals which were pre-treated with PBS only (group A) followed by EAE induction developed a moderate form of EAE, with the highest average DAS score (1.38) at day 31. The peak levels of the DAS scores of animals of groups B (1.63) and C (1.75), which were pre-treated by oral administration of different doses of 100 and 500 μg PLP-peptide prior to EAE induction, were reached on day 34 and 32 respectively. During the entire course of the disease, the average DAS scores of the animals of groups B (100 μg) and C (1000 μg) were higher as compared to the average DAS scores of the animals of group A. The oral administration of PLP-peptide simultaneously with *Lactobacillus casei* (groups E (100 μg) and F (500 μg)) resulted in a synergistic enhancement of EAE (E and F vs D). Peak levels of average DAS scores of the animals of group E (3.14) and F (3.38) were higher and were reached earlier as compared to groups B (1.63) and C (1.75), which received the same amount of peptide but no *Lactobacillus casei* (table 2).

Table 2
Oral administration and EAE induction.

Group code:	A	B	C	D	E	F
Oral administration						
Solvent (500 μl):	PBS	PBS	PBS	PBS	PBS	PBS
PLP-peptide (μg):	0	100	500	0	100	500
Dose *Lactobacillus* (CFU):	0	0	0	5.10^8	5.10^8	5.10^8
Number of administrations:	5	5	5	5	5	5
EAE induction						
PLP-peptide (μg):	150	150	150	150	150	150
Maximum DAS levels:	1.38	1.63	1.75	2.71	3.14	3.38
Maximum levels on day:	31	34	32	32	29	30

Discussion

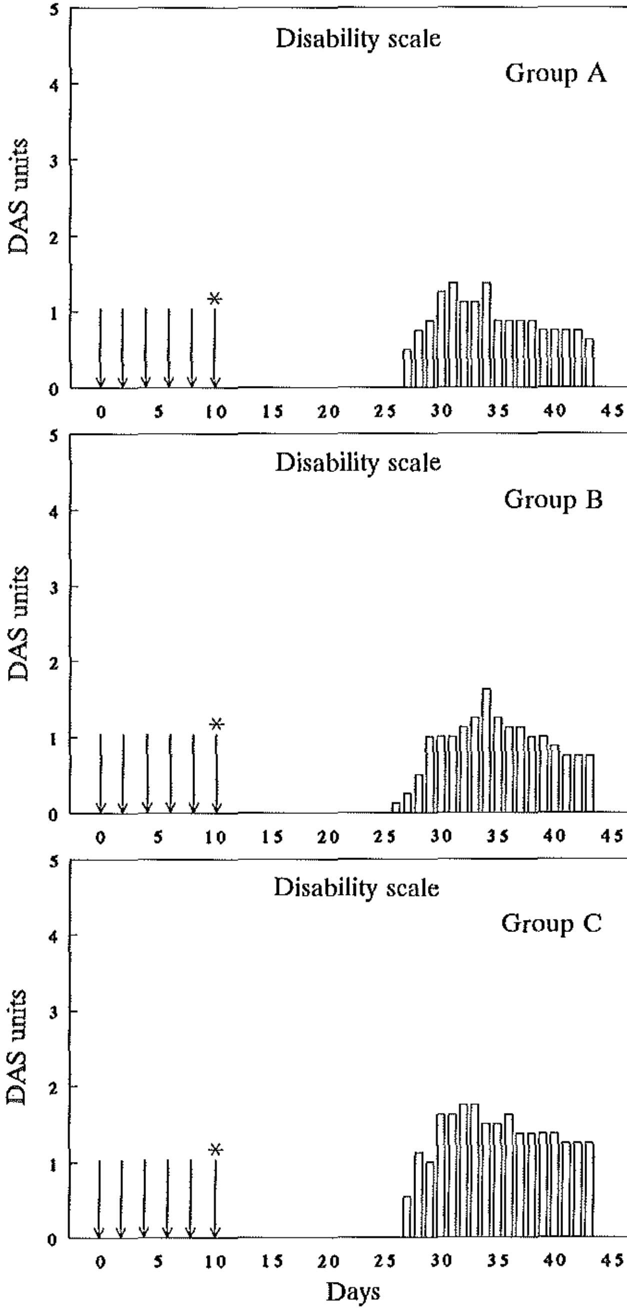
In this study we show that oral administration of β-galactosidase producing Lactobacilli, with low adjuvant properties, induces a state of peripheral immune suppression. This state of peripheral immune suppression, which is reflected by a reduced anti-β-galactosidase antibody response after a subsequent booster injection with β-galactosidase, is also significant after adoptive transfer of the spleen cells to naive recipient mice. Furthermore, the oral administration of Lactobacilli with high adjuvant properties, together with EAE inducing peptides, results in stimulation of the immune system as monitored by EAE disease enhancement.

The oral delivery of the intact protein β-galactosidase by $LP80^+$, prior to i.p. booster I and II injections with β-galactosidase, induces a reduction in anti-β-galactosidase responsiveness. This may be interpreted as (incomplete) tolerance induction. The average anti-β-galactosidase antibody response in animals which received 50 μg transformed $LP80^+$ was reduced 64% after the second booster β-galactosidase injection, as compared to animals not receiving tolerance inductive pretreatment. The average anti-β-galactosidase antibody responses were reduced 52%, 27% and 39% in the animals which received orally 100 μg, 500 μg and 1000 μg of transformed $LP80^+$ respectively, after the second booster β-galactosidase injection. The observation that small amounts of orally administered antigen were more efficient in inducing non-responsiveness as compared to large amounts of oral

---

Figure 3 (next page)
Oral administration of a synthetic epitope mildly enhances EAE induction.
After oral administrations and EAE induction, the severity of clinical signs was evaluated every day. The results of the mice of group A, B and C are expressed as average DAS units per day, in the upper, middle and lower panel respectively. Oral administrations were performed on day 0, 2, 4, 6 and 8 (arrows). EAE was induced on day 10(*).

Figure 3



administered antigen suggests that there exists a low zone tolerance for β-galactosidase. In the present experiments only a state of incomplete low zone tolerance was observed. This might be explained by differences in antigen processing of intact Lactobacilli with intracellular β-galactosidase and processing of free soluble β-galactosidase derived from Lactobacilli degraded in the gastro intestinal tract which may lead to differences in the induction of immunological unresponsiveness.

Stokes et al. (1983) have suggested that oral immunization, i.e. the development of an antibody response, requires a fast antigen uptake by the intestinal epithelium, whereas oral tolerance requires a gradual antigen delivery. The transformed *Lactobacillus plantarum* strain used in our experiments produces β-galactosidase in the cytoplasm. The antigen is most likely released from the cytoplasm upon death and cytolysis of the strain in the gastrointestinal tract. Possibly presentation of the antigen by the $LP80^+$ in our experiment may lead to a relatively fast antigen uptake and therefore might be a reason for the observed incomplete tolerance as well. However, various other methods of antigen delivery (presentation) by micro-organisms can be envisaged to realize a differential uptake of antigen: a) the antigen expressed by *Lactobacillus* and subsequently secreted in the gastrointestinal tract and b) the antigen is expressed on the surface of the *Lactobacillus* and is presented either by live or dead organisms to the gut associated lymphoid tissues. Experimental evaluation of these modes of antigen presentation by orally administered transformed microorganisms for induction of peripheral tolerance is presently investigated.

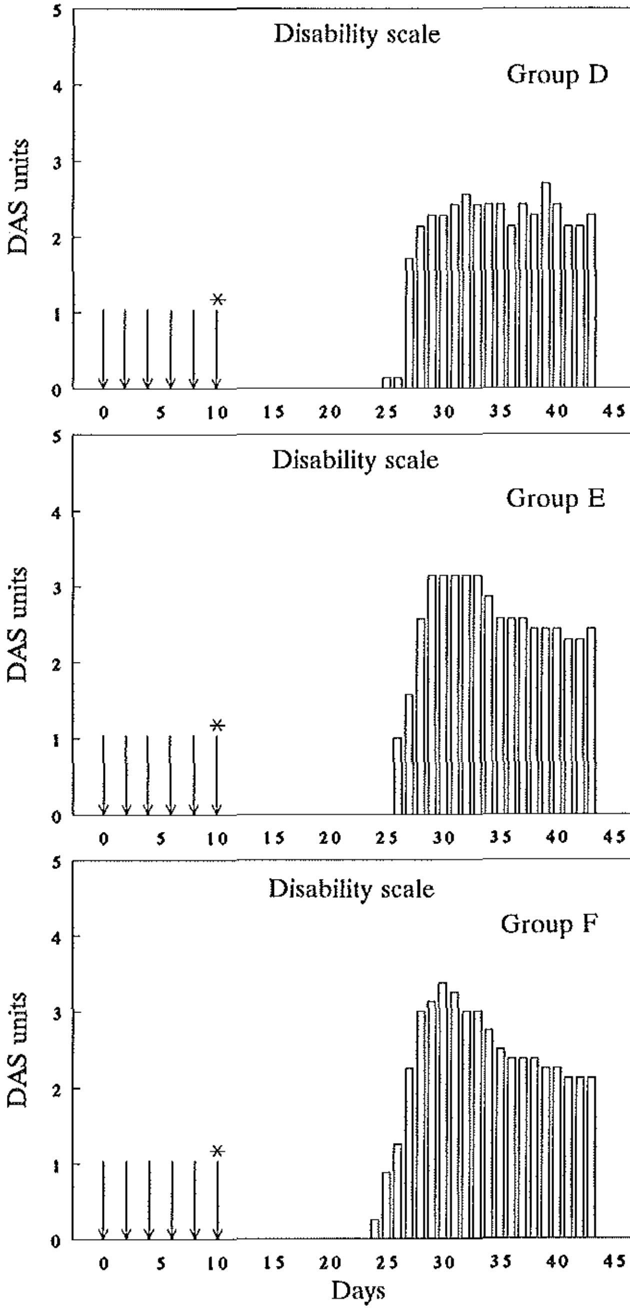
Another factor which may influence the completion of tolerance induction is the age (12-15 weeks) of the animals. Faria et al. (1993) showed that $B6D2F_1$, C3H/HeJ and A/J mice were highly susceptible to induction of oral tolerance against ovalbumin at 8 weeks of age. In contrast, no oral tolerance induction was observed in A/J mice and only a partial tolerance was induced in the $B6D2F_1$ and C3H/HeJ mice, which were 24 weeks old. Also in C57Bl/6J mice, age has been shown a limiting factor for oral tolerance induction as well (Rios et al., 1988). These results suggest that, dependent on genetic background, there is a certain time window in the ontogeny in which the mice are most susceptible to induction of oral tolerance. The period in which the mice are most susceptible to oral tolerance induction in general is found shortly after immunological maturation (Hanson, 1981). Possibly oral administration of $LP80^+$ to younger mice (8-10 weeks), instead of to young adult mice (12-15 weeks) as in our experiment, might improve tolerance induction.

Figure 4 (next page)

Oral administrations of *Lactobacillus casei* synergistically enhances EAE responsiveness.

After oral administrations and EAE induction, the severity of clinical signs was evaluated every day. The results of the mice of group D, E and F are expressed as average DAS units per day, in the upper, middle and lower panel respectively. Oral administrations were performed on day 0, 2, 4, 6 and 8 (arrows). EAE was induced on day 10(*).

Figure 4

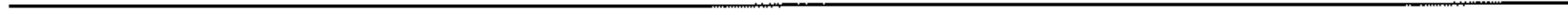


We have investigated the effect of oral administration of EAE inductive peptide with and without high adjuvant *L.casei* on EAE induction. It was shown that oral administration of low doses of 100 μg and 500 μg peptide, prior to disease induction, did not induce tolerance for EAE (group B and C). At least two reasons might explain why tolerance induction was not accomplished in these mice: a) the high doses of antigen used for oral pre-treatment and b) the choice of an encephalitogenic PLP-epitope. It may be assumed that the amount of PLP-peptide which we have used for oral administration is still too large to be efficient for low zone tolerance induction. In the experiments using $LP80^+$ only the smallest amounts of β-galactosidase producing $LP80^+$ were efficient in tolerance induction. These mice received an estimated amount of 0.1 μg β-galactosidase, which is at least a factor 1000 less as compared to the lowest amount of PLP-peptide administered to the SJL/j mice. Furthermore, taking the molecular weight of both β-galactosidase and PLP-peptide in consideration, the amount of β-galactosidase may be reduced with another factor 200 as compared to the amount of PLP-peptide used for pre-treatment. The choice of the encephalitogenic PLP epitope for oral administration may be due to the failure to induce tolerance as well. Both in Lewis rat and SJL/j mice a number of peptides have been described which can induce oral tolerance against EAE induction. These include the non-encephalitogenic tolerizing decapeptide from MBP (Higgins and Weiner, 1988) and a variety of other MBP sequences (Avrilionis and Boggs, 1991; Naiki et al., 1991; Su and Sriram, 1991; Weiner et al., 1992). In subsequent studies, the synergistic effects of oral administration of tolerizing epitopes on EAE will be studied.

Oral administration *L.casei* (group E and F) prior to EAE induction resulted in a considerable EAE enhancement (figure 4) as compared to the course of disease of animals which were not orally pretreated with *L.casei* (figure 3). The severity of clinical signs was even enhanced when *L.casei* was orally administered without peptide (group D vs A). Therefore the enhancement of disease is most likely due to the high adjuvant properties of this *Lactobacillus* strain. Some *Lactobacillus* strains can exert non-specific immunostimulatory effects (Bloksma et al., 1979)(adjuvanticity) which seems mainly due to wall components of the bacteria (Kato et al., 1984; Yokokura et al., 1986). However *Lactobacillus* do not produce LPS and the mechanism (component) of adjuvant activity of *Lactobacillus* is still unknown (Claassen et al., 1994). The adjuvant properties of *Lactobacillus* may influence whether the immune system is activated or tolerized, upon oral delivery of *Lactobacillus* and antigens. In general, immune responses are enhanced by adjuvant (Claassen and Boersma, 1992). On one hand, Goverman et al. (1993) showed that spontaneous experimental autoimmune encephalomyelitis (EAE), can only develop in myelin basic protein (MBP) specific T-cell receptor transgenic mice when the animals were housed in a non-sterile facility and not when they were kept under specific pathogen free conditions. These findings suggest that the presence of specific bacteria in the gastro-intestinal tract contributes to the activation of the

immune system of EAE animals. On the other hand it has also been shown that colonization of the gastro-intestinal tract by lipopolysacharide (LPS) producing micro-organisms is one of the requirements for oral tolerization (Wannemuhler et al., 1982). Oral tolerance to MBP is enhanced by the oral administration of LPS, possibly through the production of specific cytokines, i.e. TGF-β and IL4 (Khoury et al., 1992). Furthermore it was shown that certain bacteria, i.e. *Bordetella pertussis* and *Mycobacterium tuberculosis* can play a beneficial role in the induction of T cell mediated suppression (Lehmann and Ben-Nun, 1992).

In conclusion, this study shows that partial induction of peripheral tolerance in young adult mice, can be established after the oral administration of β-galactosidase producing *Lactobacillus* LP80$^+$ with low adjuvant properties. In contrast, the use of *L.casei* with high adjuvant properties, mixed together with soluble antigen, resulted in enhancement of the immune response. The results of this study indicate that *Lactobacillus* are potential candidates for the delivery of immune stimulating signals as well as for the induction of peripheral tolerance against putative autoantigens in human autoimmune diseases.



CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

Introduction

Multiple sclerosis (MS) presents itself as an inflammatory autoimmune disease which is restricted to the central nervous system (CNS). In the inflammatory reaction mononuclear cells, antigen presenting cells (APC), B-cells and regulatory and effector T-cells are involved. Animal studies revealed that also other cells may be involved in the immune response. MHC class II expression has been demonstrated on meningeal cells, perivascular cells, astrocytes and microglia cells (Hickey and Kimura, 1988; Vass and Lassmann, 1990). Furthermore, astrocytes and glial cells have been shown to produce cytokines (Robbins et al., 1987).

Despite intensive scientific and clinical research on MS, the etiology and pathogenesis is still unknown. Several hypotheses on the etiology of MS have been postulated; MS may be induced by an infectious organism, an autoantigen or an antigen mimicking a self-component. However until now no single infectious organism, autoantigen or antigen has been linked to the disease. The "consensus" hypothesis on the etiology of MS is that a combination of environmental factors in a genetically susceptible subject may lead to the induction of an autoimmune response.

Based on the premise that MS is mediated by an aberrant immune response most approaches to prevent MS, which were developed so far, are related to intervention or enhancement of certain immunological processes. This chapter will discuss the relevance of the experiments described in this thesis in relation to recent developments concerning immuno-intervention in MS.

B-cell specificity

In most MS patients an elevated level of intrathecally synthesized immunoglobulins in the cerebrospinal fluid (CSF) is observed (Walsh and Tourtelotte, 1983). These antibodies show a polyclonal banding pattern when separated by isoelectric focusing, indicating an antibody response based on a limited number of specificities. Some of the polyclonal antibodies are directed against CNS autoantigens, however the majority of the antibody specificities still remain obscure. It has been suggested that a part of the antibodies are directed against idiotypes of the autoantigen antibodies. According to the network theory of Jerne (1975) it is conceivable that anti-idiotype antibodies may down regulate the autoantibody response without undesirable side effects. Down regulation of the autoantibody response based on the production of anti-idiotypic antibodies may contribute to therapy of MS. Whether this anti-idiotype antibody based method can be applied to

decrease autoantibody levels remains to be investigated.

Antibodies against a variety of CNS antigens have been detected also in sera of MS patients. However, antibodies directed against CNS antigens have been detected in the CSF and sera of healthy subjects as well. In most studies autoantibody levels in sera and CSF of MS patients were increased as compared to the autoantibody levels in healthy subjects. In *chapter 2* we have shown that the majority of the AFCs detected in brain tissue sections was directed against MBP. Furthermore we have shown that the presence of MBP specific antibody forming cells in CNS tissue sections is specific for MS patients. Anti-myelin basic protein (MBP) antibody forming cells (AFCs) were not detected in CNS tissue sections of healthy subjects nor in CNS tissue sections of patients with other neurological diseases. This finding indicate that the antibody response to myelin basic protein is important in MS immunopathology. This was confirmed in a study performed recently by Warren et al. (1994). In a panel of 116 chronic progressive MS patient it was shown that 111 had anti-MBP titers in the cerebrospinal fluid, while 173 of 180 relapsing MS patients had anti-MBP antibody in the CSF. Although these findings are highly suggestive for an important role of MBP as antigen it can not be concluded that MBP is the primary target antigen. One of the problems in identifying the target antigen in autoimmune diseases in general is the occurrence of antigen diversification of B-cell responses to other antigens, after a primary immune response which triggered the disease.

B-cell activation

The development of an autoantibody response in MS is generally explained as a secondary phenomenon due to the release of autoantigens to the circulation. Autoantigens have been detected in serum and urine of MS-patients (Whitaker, 1977; 1987). Autoantigens in the circulation will eventually arrive in the peripheral immunological tissues and will be presented to B-cells. Guided by autoantigen specific T-cells these cells will proliferate and differentiate. Subsequently, the autoantigen specific AFCs will reach the blood brain barrier due to normal immune-surveillance and in case of local expression of adherence molecules, the autoantigen specific AFCs will enter the CNS tissues. On the other hand it can not be excluded that the B-cell response is not "autoantigen driven" as is described above, but that an additional "defective" control mechanism, e.g. the neuro-endocrine system, is due to the increased number of autoreactive B-cells in MS patient sera and CSF.

In addition, a damaged blood brain barrier will facilitate a non-antigen specific entrance of B-cells into the CNS tissues. Due to presence of autoantigens especially the autoantigen reactive B-cells will be activated. In *chapter 3* we have demonstrated that gp39 positive T-cells are localized in the same affected region of the CNS tissues as the AFCs specific for MBP. This observation indicate that anti-MBP AFCs may be activated locally. Although the observed autoantibody response

in MS patients is generally explained as a secondary phenomenon, it should be emphasized that "secondary" autoantibodies may contribute to immune mediated demyelination as well.

It can not be excluded however, that the autoreactive B-cell response in MS is playing a primary role in the pathogenesis. Non-antigen specific homing of lymphocytes to the CNS tissues takes place even in healthy, normal unaffected brain. Adhesion molecules expressed by immune-competent cells are candidates to guide the cells through the blood brain barrier and subsequently into the CNS. Several families of adhesion molecules are expressed on the surface of lymphocytes, having respective ligands (addressins) on endothelial cells of the blood brain barrier (Springer et al., 1987). The interaction of the lymphocyte adhesion molecules with their respective ligands not only facilitates the binding of lymphocytes to the endothelial cells but also leads to certain changes in the blood brain barrier which seems to be essential for the regulation of migration of the lymphocytes (Akiyama et al., 1989). This indicates that B-cells may enter the CNS even before the blood brain barrier is damaged and therefore could play a role even in the initial events leading to demyelination.

B-cell functions

Studies with EAE animals suggest an important role for autoantibodies and autoantibody forming cells in the process of demyelination. Antibody deficient rats fail to develop clinical or histological evidence of EAE, when sensitized with either whole spinal cord homogenate or purified myelin basic protein (Willenborg and Prowse, 1983). Intravenous injection of monoclonal antibodies against myelin oligodendrocyte glycoprotein in EAE in Lewis rats resulted in augmentation of both clinical signs, inflammation and demyelination (Lassmann et al., 1988). Furthermore, in the presence of anti-myelin oligodendrocyte glycoprotein (MOG) monoclonal antibodies 10-100 times less encephalitogenic T-cells are needed to induce EAE by passive transfer (Linington et al., 1988). The autoantibodies and autoantibody producing B-cells may be pathologically involved in demyelination in several ways. I) Autoantibodies directed against myelin components may be active in demyelination by complement mediated breakdown (Kerlero de Rosbo et al., 1990; Bradbury et al., 1984). Although, decomplementation of anti-MOG treated EAE animals only partially reduced clinical signs of the disease (Linington et al., 1990). II) Autoantibodies may be involved in demyelination by opsonization for macrophage attack (Williams et al., 1980; Ozawa et al., 1989). III) B-cells may be involved in demyelination by their antigen presenting capacity (Lanzavecchia, 1987). The membrane bound antibodies on B-cells may sequester and concentrate autoantigens which are present even in very low concentrations. The autoantigens then will be processed and presented in context of MHC class II to T-cells. This indicates that the presence of autoantibodies may focus the immune response to specific targets,

thereby inducing a very selective tissue destruction. It also indicates that the T-cell repertoire indirectly is affected by the B-cell repertoire. IV) Furthermore it was demonstrated that CNS derived immunoglobulins from MS patients are involved in the activation of a myelin associated protease acting on MBP (Kerlero de Rosbo and Bernard, 1989).

Therapeutic approaches

EAE animal studies indicate that autoantigen directed B-cells are directly related to the immunopathology of the disease. In addition, we have detected autoantigen specific B-cells in the CNS of MS-patients only (*chapter 2*). Furthermore, activation marker gp39 positive T-cells were detected (*chapter 3*) in affected areas of CNS tissues of MS-patients which indicates that at least a part of the autoreactive B-cells in MS will be activated in the CNS. These findings would strongly support the clinical use of immunosuppressive and immunomodulating therapies to reduce the levels of such putative pathogenic auto-antibodies. Therapy could be directed at the specific B-cells responsible for the production of autoantibodies as well as to the helper T-cells which are necessary for the B-cell activation. In both CSF and sera of MS patients antibodies directed against a variety of putative CNS antigens were detected (Zanetta et al., 1990a, 1990b; Endo et al., 1984; Hirsch and Parks, 1976; Link et al., 1990; Sun et al., 1991; Baig et al., 1991; Henneberg et al., 1991). From this it can be concluded that analysis of sera, CSF and immunoglobulins extracted from the brain of MS patients has failed to identify a single disease specific autoantibody population, which is directed against a CNS autoantigen. Therefore methods for the selective elimination of autoantigen specific B-cells will not be helpful in MS until the major primary target antigen is identified. In view of the heterogenous antibody specificity only therapeutical methods with a broad-spectrum (aspecific) character like immunosuppressive drugs can be applied. However, in general immunosuppressive drugs do not cross the blood brain barrier. Therefore these drugs are not effective against B and T-cells within the CNS. On the other hand if cells which are responsible for the antibody formation continuously enter the CNS, it may be possible to suppress the antibody production in the CNS indirectly by manipulating the cells of the peripheral immune system. In view of this, the effect of plasmapheresis on the local CNS autoantibody levels and as a consequence on the development of disease, has to be evaluated.

If vascular addressins play an important role in lymphocyte traffic between blood circulation and the CNS compartment in MS, they may be considered to be important in CNS specific anti-inflammation therapy development. A high level of expression of vascular addressins was detected in venules in the proximity of lesions in the CNS of MS-patients (Raine et al., 1990). Therefore the administration of soluble factors which inhibit the expression of adhesion molecules or block the adhesion molecules is a promising approach to interfere in the blood brain barrier

traffic in MS. Yednock et al. (1992) already showed that the administration of antibodies directed against integrin $\alpha 4\beta 1$ prevented the accumulation of lymphocytes in the CNS and inhibited the development of experimental autoimmune encephalomyelitis (EAE).

EAE is transferred by autoantigen reactive T-cells to naive recipients. MBP reactive T-cell lines induce demyelination when added to myelinated CNS cultures (Lyman et al., 1988). Other T-cells can exert an antigen specific cytotoxicity against antigen presenting cells like astrocytes and cerebral endothelial cells (Sun and Wekerle, 1986; Risau et al., 1990). Although as yet the definite proof lacks that cell mediated activity is the crucial pathological event in man as well, the findings in the EAE animal model and the *in vitro* experiments with human T-cells, strongly support the idea that MS is a T-cell mediated disease. As a consequence experimental MS therapy developed sofar, was focused on the reduction or modulation of the cell mediated activity. Broad range effective agents, like cytokines, anti-cytokine antibodies, soluble cytokine receptors, anti-lymphocyte surface antigen antibodies and immunotoxins have been used to reduce T-cell (mediated) autoimmune responses in MS. However, as with the modulation of the antibody mediated activity it is not clear in what way the modulation of peripheral T-cell activity influences the local CNS T-cell activity. Until now it was shown that the systemic administration of these broad range agents has a relatively low effect on the development of the disease. In addition, it is not clear which subset(s) of T-cells are pathologically most important and which antigens are the target antigens. Therefore broad range immunosuppression seems to be the only way to modulate T-cell mediated activity in MS. Many immunosuppressive drug have anti-inflammatory effects as well and the use of this kind of drugs would eliminate the need to identify the target antigen(s). On the other hand, the use of anti-inflammatory drugs affects all inflammatory responses, also those responses which are necessary for the normal body defense mechanisms. Furthermore, if one supposes a viral ethiology of MS, an appropriate inflammatory immune response is essential to prevent further spreading of the virus (development of the disease).

A more specific immune intervention can be obtained by blocking the APC/T-cell interaction. This can be achieved by administration of agents which target on either the MHC products or by agents which target on the T-cell receptor (TCR), such that encephalitogenic T-cells can not be triggered. A strain dependent limited $V\beta$ gene usage by cells specific for MBP has been identified in both rats and mice. EAE can be prevented by pretreatment with monoclonal antibodies directed against the $V\beta 8$ coded TCR chain. Furthermore EAE remission is correlated with the development an anti- $V\beta 8.2$ antibody response. Also direct immunization with a $V\beta 8.2$ peptide, representing the antibody epitope, resulted in prevention of disease induction (Hashim et al., 1992). Data have been presented that class I $CD8^+$ T-cells, reactive with the TCR peptides, are responsible for the protection against EAE

disease induction (Offner et al., 1991). On the other hand it was shown that $CD4^+$ T-cells secrete the inhibitory TGF-β and IL-4 cytokines upon exposure to a Vβ8 peptide (Karpus et al., 1992). Furthermore, it was shown that TCR gene Vβ17 was predominantly used by human T-cells reactive with MBP fragment 84-102 (Wucherpfennig et al., 1990). However, with some exceptions, the Vβ usage will vary between individuals (Ben-Nun et al., 1991), this makes the identification of a consensus target molecule, which can be used to block the TCR in more than one subject, very difficult. Therefore, the TCR blockade therapy seems to be useful in individual patients only, presuming that the administration of these peptides in human can reduce the activity of autoreactive T-cells. The usage of peptide mixtures could make the TCR-blockade therapy even more widely applicable.

An alternative way of blocking cellular interactions and as a consequence the activation of cells is the use of antibodies. In *chapter 3* it was demonstrated that the administration of anti-gp39 monoclonal antibodies, prevented the development of EAE. Even the administration of anti-gp39 after disease onset, shortly before maximum disability score was reached, lead to dramatic disease reduction. These results indicate that gp39-CD40 interactions are required to maintain EAE. However, it is unknown through which mechanism gp39 and CD40 are involved in the maintenance of the disease. Several possible mechanisms can be distinguished. I) As was discussed before (see B-cell function) autoantibodies produced by CD40 positive B-cells, may be involved in the process of demyelination in many ways. II) Macrophages expressing CD40 may be activated by $gp39^+$ T-cells as well. Macrophages can be involved in demyelination by the production of cytotoxic and/or inflammatory cytokines and cytotoxic oxygen radicals. III) The $gp39^+$ T-cells may be involved in pathology of the disease due to the production of a variety of inflammatory cytokines. In *chapter 3* we have shown that $gp39^+$ T-cells are present in CNS tissue sections of MS patients. This finding indicate that gp39/CD40 interactions are possibly involved in MS immunopathology. Therefore, in analogy to the results obtained in EAE, the blockade of cognate gp39-CD40 interactions might be considered to induce a long term antigen specific suppression in MS demyelination as well. However, since the gp39 membrane marker is expressed on all activated T-cells, anti-gp39 treatment may be considered to be a therapeutic method with a limited specificity only. An alternative approach to induce long term antigen specific suppression is the induction of oral tolerance.

Tolerance induction

In normal healthy subjects food antigens normally do not provoke an immunological reaction. This implicates that feeding of autoantigens to humans, under certain conditions, may be expected to induce a state of antigen specific tolerance, as they do in animals. Therefore, the most promising non-toxic, immuno-specific therapy is tolerance induction by oral administration of autoantigens.

However, an essential requirement for this kind of therapy is the identification of the autoantigen(s). In EAE animals, the oral administration of MBP, prior to disease induction and subsequent suppression of disease in treated animals induced an increased local production of the inhibitory cytokine TGF-β (Khoury et al., 1992). Under certain conditions, oral tolerance to MBP may be mediated by clonal deletion, rather than by active suppression due to the action of cytokines. After oral administration most antigens are processed first by intestinal enzymes and secondly by intestinal epithelial antigen-presenting cells, which will probably present the tolerogenic epitopes to intraepithelial lymphocytes. The activated or tolerized lymphocytes migrate via the mesentherial lymph nodes and thoracic duct to the spleen where they may mediate induction of anergy (Whitacre et al., 1989; 1990).

Although the induction of oral tolerance in animals is most promising and is considered as a possible method for MS-therapy as well, oral tolerance induction therapy has still some disadvantages. Oral administration of autoantigens may enhance an already established systemic immune response (Hanson et al., 1979). Dependent on dose of antigen, administration regimen, delivery system and adjuvant properties of the delivery system it is possible to induce a systemic immunological memory formation, following the same route as is used for tolerance induction (*chapter 4.3*). This indicates that secondary conditions will to a large extent determine the outcome of tolerance induction protocols. As was shown in *chapter 4.4*, the oral administration of PLP synthetic epitopes together with *Lactobacillus casei* prior to EAE induction in naive SJL/J mice resulted even in a significant enhancement of the immune response.

Antigens may exhibit inductive as well as tolerogenic epitopes. Therefor an alternative approach to reduce the possibility of disease exacerbations after oral administration of autoantigens is the use of selected tolerogenic synthetic peptides fragments, which are not autoimmunogenic by itself. Both in Lewis rat and SJL/J mouse EAE models a number of peptides, related to myelin proteins, have been described which can be applied to induce oral tolerance against subsequent EAE induction. These peptides include the non-encephalitogenic tolerizing decapeptide from MBP and a variety of other MBP sequences (Higgins and Weiner, 1988; Su and Sriram, 1991; Avrilionis and Boggs, 1991; Weiner et al., 1992; Naiki et al., 1991). The production of such peptides by live transformed microorganisms, which are administered orally, would prevent their complete degradation by intestinal enzymes and would facilitate a continuous delivery of peptide to the intestinal epithelium as well. We have shown that GRAS *Lactobacillus* species are convenient microorganisms to a) present and deliver antigens to the immune system via the oral route (*chapter 4.2*) and b) dependent on the adjuvant properties of the *Lactobacillus* strain, Lactobacilli can induce at least a transient state of peripheral tolerance when administered orally (*chapter 4.4*). Therefore, the oral administration of *Lactobacillus* strains, genetically transformed to produce and secrete tolerogenic epitopes, seems to be a most promising approach to induce peripheral tolerance without disease

exacerbations.

The ultimate intention of tolerance induction by the oral administration of autoantigens is the induction or re-establishment of natural tolerance in patients with disease. Until now most experiments performed with animal autoimmune diseases were based on the modification of the immune response by oral administration of autoantigens before disease induction by the parental route (Nussenblat et al., 1990). Oral administration of MBP in Lewis rats during the pre-clinical period of EAE, did lower the incidence and severity of clinical signs of EAE, but was not able to induce a state of tolerance similar to that observed after oral administration of MBP before disease induction (Higgins and Weiner, 1988). However, the induction of oral tolerance to MBP can be enhanced by the oral co-administration of lipopolysacharide (LPS) as an adjuvant (Khoury et al., 1990), which suggest that microorganisms which express LPS may especially be convenient for the oral delivery of tolerogenic epitopes in order to induce tolerance against putative CNS autoantigens in MS.

Concluding remarks

The experiments to study the involvement of autoantibody forming cells in immunopathology of MS and the experiments concerning the use of microorganisms as antigen presenting vehicle by the oral route have raised many new scientific and clinical questions. In the experiments presented in *chapter 2*, we have limited ourselves to the detection of antibody forming cells directed against only one putative CNS autoantigen. One specimen of microorganisms was applied to investigate its putative use as an oral delivery system (*chapter 4*). Due to these limitations and to the fact that this field is evolving rapidly it is very difficult to draw definite conclusions.

However, the presence of myelin basic protein antibody forming cells and B-cell activation-marker-positive T-cells in affected areas of MS patients as was demonstrated in this thesis, together with the large body of literature on antibody mediated demyelination in *in vitro* or *in vivo* animal experiments strongly suggests that myelin basic protein specific antibodies are involved in MS immunopathology. The mechanism of action being still unknown. Similarities between the immunological anomalies associated with MS and the T-cell mediated demyelinating animal model of experimental autoimmune encephalomyelitis, has resulted in focusing of therapeutical approaches on the T-cell. Based on the major role of T-cells (help to other cells (B-cells), cytotoxicity, suppression) in many immunological processes in general, it seems worthwhile to block one or more T-cell related functions in a non-antigen specific way, i.e. irrespective of the identity of the putative target autoantigen(s). As we have demonstrated in this thesis (*chapter 3*) the blockade of a T-cell membrane marker gp39 by the administration of monoclonal antibodies was successful in reduction of disease in EAE animals, even if the

antibodies were administered after disease onset. These findings suggest that the blockade of cellular interactions, especially if the blockade can be focused within the CNS, may be a useful method to interfere in MS immunopathology as well.

The ultimate goal in MS therapy development will be the restoration of the normal adult immune system by the induction of tolerance. A promising approach to induce tolerance is the administration of tolerogenic epitopes of selected auto-antigens by the oral route. In this thesis (*chapter 4*) we have demonstrated that peripheral tolerance can be induced by the oral administration of genetically modified *Lactobacillus* producing antigen. This approach is may be useful to provide specific immunotherapy in MS patients as well.

However, it should be emphasized that the human immune system provides several mechanisms which could damage the central nervous system in many ways. In other words, numerous different antigens can be the target of different effector cells which can produce various effector molecules at the same time. As a consequence, the blockade of one single cell interaction or the induction of tolerance against one single putative antigen may have some beneficial effect on the disease, however these approaches will never cure the disease.

REFERENCES

Adams RD and Sidman RL. Introduction to neuropathology. New York, McGraw-Hill, 1968.

Adams JM, Brooks MB, Fisher ED and Tyler CS. Measles antibodies in patients with multiple sclerosis and with other neurological and non-neurological disorders. Neurology 20:1039, 1970.

Adams CW. The general pathology of multiple sclerosis: Morphological and chemical aspects of the lesions. In: Multiple Sclerosis. Pathology, diagnosis and management. Hallpike JF, Adams CWM, Toutelotte WW, Eds. London, Chapman and Hall, 203, 1983.

Aita JF, Bennett DR, Anderson RE and Zitter F. Cranial CT appearance of acute multiple sclerosis. Neurology 28:251, 1978.

Akiyama SK, Yamada SS, Chen WT and Yamada KM. Analysis of fibrinonectin function with monoclonal antibodies: Roles in cell adhesion, migration, matrix assembly and cytoskeletal organisation. J Cell Biol 109:863, 1989.

Alderson MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC and Spriggs MK. CD40 expression by human monocytes: Regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med 178:669, 1993.

Allison RS and Millar JHD. Prevalence and familial incidence of disseminated sclerosis. Prevalence of disseminated sclerosis in Northern Ireland. Ulster Med J 23:5, 1954.

Alter M, Kahana E and Loewenson R. Migration and risk of multiple sclerosis. Neurology 28:1089, 1978.

Alvord EC. Disseminated encephalomyelitis: Its variation in form and their relationship to other diseases of the nervous system. In: Handbook of clinical neurology. Vinken PJ, Bruyn GW and Klawans HL, Eds. Demyelinating diseases. Koetsier JC, Ed. Amsterdam, Elsevier, 3:467, 1985.

Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson DM, Gimpel SD, Davis-Smith T, Maliszewski CR, Clark EA, Smith CA, Grabstein KH, Cosman D and Spriggs MK. Molecular and biological characterization of a murine ligand for CD40. Nature 357:80, 1992.

Arnason BGW and Reder AT. Interferons and multiple sclerosis. Clinical Neuropharmacology 17:495, 1994.

Askelöf P, Rodmalm K, Wrangsell G, Larsson U, Svenson SB, Cowell JL, Undén A and Bartfai T. Protective immunogenicity of two synthetic peptides selected from the amino acid sequence of Bordetella pertussis toxin subunit S1. Proc Natl Acad Sci 87:1347, 1988.

Avrilionis K and Boggs JM. Suppression of experimental allergic encephalomyelitis by the encephalitogenic peptide, in solution or bound to liposomes. J Neuroimmunol 35:201, 1991.

Barefoot SF and Klaenhammer TR. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol 45:1808, 1983.

Batchelor JR, Compston A and McDonald WI. The significance of the association between HLA and multiple sclerosis. Br Med Bull 34:279, 1978.

Baig S, Olsson T, Yu-Ping J, Höjeberg B, Cruz M and Link H. Multiple Sclerosis: Cells secreting antibodies against myelin-associated glycoprotein are present in cerebrospinal fluid. Scand J Immunol 33:73, 1991.

Bauer J, Sminia T, Wouterlood FG and Dijkstra CD. Phagocytic activity of macrophages and microglial cells during the course of acute and chronic relapsing experimental autoimmune encephalomyelitis. J Neurosci Res 38:365, 1994.

Ben-Nun A and Cohen IR. Spontaneous remission and acquired resistance to EAE are associated with suppression of T cell reactivity: Suppressed EAE effector cells recovered as T cell lines. J Immunol 128:1450, 1982.

Ben-Nun A, Liblau RS, Cohen L, Lehmann D, Tournier-Lasserve E, Rosenzweig S, Jingwu Z, Raus JCM and Bach MA. Restricted T-cell receptor Vβ gene usage by myelin basic protein-specific T-cell clones in multiple sclerosis: Predominant genes vary in individuals. Proc Natl Acad Sci USA 88:2466, 1991.

Bhatnagar PK, Mao SJT, Gotto Jr AM and Sparrow JT. The application of an improved solid phase synthetic technique to the delineation of an antigenic site of apolipoprotein A-II. Peptides 4:343, 1983.

Black R, Levine MM, Young C, Rooney J, Levine S, Clements ML, O'Donell S, Hughes T and Germainer R. Immunogenicity of Ty21a attenuated *Salmonella typhi* given with sodium bicarbonate or in enteric-coated capsules. Dev Biol Stand 53:9, 1983.

Bloksma N, De Heer E, Van Dijk H and Willers JM. Adjuvanticity of lactobacilli. Differential effects of viable and killed bacteria. Clin Exp Immunol 37:367, 1979.

Boersma WJA, Claassen E, Deen C, Gerritse K, Haaijman JJ and Zegers ND. Antibodies to short synthetic peptides for specific recognition of partly denatured protein. Analytica Chimica Acta 213:187, 1988.

Boersma WJA, Deen C, Gerritse K, Zegers ND, Haaijman JJ and Claassen E. Anti-peptide antibodies as subclass specific reagents: Epitope mapping of human IgG2. Prot Biol Fluids 36:161, 1989.

Boersma WJA, Haaijman JJ and Claassen E. Use of synthetic peptide determinants for the production of antibodies. In: Immunohistochemistry 2nd Ed. Cuello AC, Wiley & Sons, 1, 1993.

Boersma WJA, Zegers ND, Van den Bogaerd A, Leer RJ, Bergmans A, Pouwels PH, Posna M and Claassen E. Development of safe oral vaccines based on *Lactobacillus* as a vector with adjuvant activity. Proc ICHEM 2nd Internatl Congr Biotech, UK, Brighton, 43, 1994.

Bokhout BA, Van Gaalen C and Van der Heijden J. A selected water-in-oil emulsion: Composition and usefulness as an immunological adjuvant. Vet Immunol Immunopathol 2:491, 1981.

Bondi A, Chieregatti G, Eusebi V, Fulcheri E and Bussolati G. The use of β-galactosidase as a tracer in immunocytochemistry. Histochem 76:153, 1982.

Boorsma DM. Direct immunoenzyme double staining applicable for monoclonal antibodies. Histochem 80:103, 1984.

Boorsma DM, Claassen E, Kors N, De Haan P and Van Rooijen N. Detection of specific anti-hapten-antibody producing hybridoma cells in tissue sections of spleen and liver, by hapten-enzyme conjugates in an immunoenzyme approach. Hybridoma 5:347, 1986.

Boorsma DM. Conjugation of antibodies and enzymes. In: Molecular Neuroanatomy. Van Leeuwen, Buijs, Pool and Pach, Eds. Elsevier Science Publishers BV, Biomedical Division, 281, 1988.

Bourdette DN, Driscoll BF, Seil KJ, Kies MW and Alvord EC. Severity of demyelination *in vivo* correlates with serum myelination inhibition activity in guinea pigs having a new form of experimental allergic encephalomyelitis. Neurochem Pathol 4:1, 1986.

Bradbury K, Aparicio SR, Sumner DW and Bird CC. Role of complement in demyelination *in vitro* by multiple sclerosis serum and other neurological disease sera. J Neuro Sci 65:293, 1984.

Brod SA, Al-Sabbagh A, Sobel RA, Hafler DA and Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin antigens: IV. Suppression of chronic relapsing disease in the Lewis rat and strain 13 guinea pig. Ann Neurol 29:615, 1991.

Brown AM and McFarlin DE. Relapsing experimental allergic encephalomyelitis in the SJL mice. Lab Invest 45:171, 1982.

Brown AR and Claassen E. Detection of antibody, idiotype, and anti-idiotype forming cells by *in situ* immunocytochemical staining. J Immunol Meth 109:235, 1988.

Brown AR and Claassen E. B lymphocytes in the spleen: Idiotype and anti-idiotype antibody forming cells. Quantitative and qualitative aspects of an *in situ* enzymehistochemical approach. 38th Forum in Immunology. Research in Immunology 142:321, 1991.

Burks JS, Devald-Macmillan B, Jankovsky L and Gerdes J. Characterisation of corona viruses isolated using multiple sclerosis autopsy brain material. Neurology 29:547, 1979.

Burns J, Littlefield K, Gomez C and Kumar V. Assessment of antigenic determinants for the human T-cell response against myelin basic protein using overlapping synthetic peptides. J Neuroimmunol 31:105, 1991.

Burstone MS. Histochemical comparison of naphthol-phosphates for the demonstration of phosphatases. J Nat Cancer Inst 20:601, 1958.

Calder VL, Bellamy AS, Owen SJ, Lewis C, Rudge P, Davison AN and Feldmann M. Effects of cyclosporin A on expression of IL-2 and IL-2 receptors in normal and multiple sclerosis patients. Clin Exp Immunol 70:570, 1987.

Campbell PA. Immunocompetent cells in resistance to bacterial infections. Bact Rev 40:284, 1976.

Caputo D, Zaffaroni M, Ghezzi A and Cazzullo CL. Azathioprine reduces intrathecal IgG synthesis in multiple sclerosis. Acta Neurol Scand 75:85, 1987.

Carlsson P and Bratthall D. Secretory and serum antibodies against *Streptococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* in relation to ingestion of fermented milk products. Acta Odontol Scand 43:147, 1985.

Carter JL, Dawson DM and Hafler DA. Five-year experience with intensive immuno suppresssion in progressive multiple sclerosis using high-dose IV cyclophosphamide plus ACTH. Neurology 36:284, 1986.

Cash E, Weerth S, Voltz R and Kornhuber M. Cells of cerebrospinal fluid of multiple sclerosis patients secrete antibodies to myelin basic protein *in vitro*. Scand J Immunol 35:695, 1992.

Catalano Jr LW. Herpes virus hominis antibody in multiple sclerosis and amyotropic lateral sclerosis. Neurology 22:473, 1972.

Challacombe SJ and Tomasi TB. Systemic tolerance and secretory immuninity after oral immunization. J Exp Med 153:1459, 1980.

Charbit A, Boulain JC and Hofnung MA. A genetical procedure devised to expose a given epitope at the cell surface of the bacterium *Escherichia coli*. Perspectives. CR Acad Sci 302:617, 1986.

Chassy BM. Prospects for improving economically significant *Lactobacillus* strains by genetic technology. Trends Biotechnol 3:273, 1985.

Chassy BM. Prospects for the genetic manipulation of lactobacilli. FEMS Microbiol Rev 46:297, 1987.

Chassy BM and Flickinger JL. Transformation of *Lactobacillus casei* by electroporation. FEMS Microbiol Lett 44:173, 1987.

Chatfield SN, Stugnell RA and Dougan G. Live Salmonella as vaccines and carriers of foreign antigenic determinants. Vaccine 7:495, 1989.

Chou CHJ, Tourtelotte WW and Kibler RF. Failure to detect antibodies to myelin basic protein or peptide fragments of myelin basic protein in CSF of patients with MS. Neurology 33:24, 1983.

Christiaens H, Leer RJ, Pouwels PH and Verstraete W. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl Environm Microbiol 58:3792, 1992.

Claassen E and Van Rooijen N. TNP-enzyme conjugate for the detection of anti-TNP antibody producing cells *in vivo*. J Immunol Meth 75:181, 1984.

Claassen E and Van Rooijen N. A trinitrophenyl-poly-L-lysine-horseradish peroxidase conjugate for the detection of anti-TNP antibodies *in vivo*. J Histochem Cytochem 33:840, 1985.

Claassen E, Kors N and Van Rooijen N. Influence of carriers on the development and localization of anti-trinitrophenyl antibody forming cells in the murine spleen. Eur J Immunol 16:271, 1986a.

Claassen E, Kors N, Dijkstra CD and Van Rooijen N. Marginal zone of the spleen and the development and localization of specific antibody forming cells against thymus-dependent and thymus-independent type-2 antigens. Immunology 57:399, 1986b.

Claassen E, Adler LT and Adler FL. Double immunocytochemical staining for the *in situ* study of allotype distribution during an anti-trinitrophenyl immuneresponse in chimeric rabits. J Histochem Cytochem 34:989, 1986c.

Claassen E, Boorsma DM, Kors N and Van Rooijen N. Double enzyme conjugates, producing an intermediate color, for the simultaneous and direct detection of three different intracellular immunoglobulin determinants with only two enzymes. J Histochem Cytochem 34:423, 1986d.

Claassen E, Kors N and Van Rooijen N. Influence of carriers on the development and localization of anti-2,4,6-trinitrophenyl (TNP) antibody forming cells in the murine spleen. II. Suppressed antibody response to TNP-Ficoll after elimination of marginal zone cells. Eur J Immunol 16:492, 1986e.

Claassen E, Adler LT, Saito K, Yoshio T and Adler FL. Functional and histological studies of adoptive immunity in neonatally transplanted rabbits. Immunology 60:37, 1987a.

Claassen E, Kors N and Van Rooijen N. Immunomodulation with liposomes: The immune response elicited by liposomes with entrapped dichloromethylene diphosphonate and surface associated antigen or hapten. Immunology 60:509, 1987b.

Claassen E and Adler L. Sequential double immunocytochemical staining for the *in situ* identification of an auto-anti-allotype immune response in allotype suppressed rabbits. J Histochem Cytochem 36:1455, 1988.

Claassen E. Alkaline phosphatase-fast red fluorescence: Rediscovery of a useful label. J Immunol Meth Letter to the Editor 139:149, 1991.

Claassen E, Gerritse K, Laman JD and Boersma WJA. New immunoenzyme-cytochemical stainings for the *in situ* detection of epitope specificity and isotype of antibody forming B cells in experimental and natural (auto)immune responses in animals and man. J Immunol Meth 150:207, 1992.

Claassen E and Boersma W. Characteristics and practical use of new-generation adjuvants as an acceptable alternative to Freund's complete adjuvant. 44th Forum in Immunology. Res in Immunol 143:471, 1992.

Claassen E, Pouwels PH, Posno M and Boersma WJA. Development of safe oral vaccines based on lactobacillus as a vector. In: Recombinant Vaccines. New Vaccinology. Kurstak E, Ed. Int Comp Virology Org, Montreal. In press. 1994.

Clark EA and Lane PJ. Regulation of human B-cell activation and adhesion. Ann Rev Immunol 9:97, 1991.

Cook SD, Dowling PC and Russel WC. Neutralizing antibodies to canine distemper and measles virus in multiple sclerosis. J of the Neurological Sciences 41:61, 1979.

Cook SD, Devereux C, Troiano R, Hafstein MP, Zito G, Hernandez E, Lavenhar M, Vidaver R and Dowling PC. Effect of total lymphoid irradiation inchronic progressive multiple sclerosis. Lancet 2:1405, 1986.

Coyle PK and Procyk-Dougherty Z. Multiple sclerosis immune complexes: An analysis of component antigens and antibodies. Ann Neurol 16:660, 1984.

Craig SW and Cebra JJ. Peyer's patches: An enriched source of precursors for IgA-producing immunocytes in the rabbit. J Exp Med 134:188, 1971.

Cross AH, Hashim GA and Raine CR. Adoptive transfer of experimental allergic encephalomyelitis and localization of the encephalitogenic epitope in the SWR mouse. J Neuroimmunol 31:59, 1991.

Cruz M, Olsson T, Ernerudh J, Hojeberg B and Link H. Immunoblot detection of oligoclonal anti-myelin basic protein IgG antibodies in cerebrospinal fluid of multiple sclerosis patients. Neurology 37:1515, 1987.

Dal Canto MC and Lipton HL. Primarily demyelination in Theiler's virus infection. An ultrastructural study. Lab Invest 33:626, 1975.

Dasgupta MK, Catz I, Warren KG, McPherson TA, Dossetor JB and Carnegie PR. Myelin basic protein: A component of circulating immune complexes in multiple sclerosis. Can J Neurol Sci 10:239, 1983.

Dautigny A, Alliel PM, d'Auriol L, Pham-Dinh D, Nusbaum JL, Galibert F and Jolles P. Molecular cloning and nucleotide sequence of a CDNA clone coding for rat brain myelin proteolipid. FEBS Lett 188:33, 1985.

De Boer M, Kasran A, Kwekkeboom J, Walter H, Vandenberghe P and Ceuppens JL. Ligation of B7 with CD28/CTLA-4 on T cells results in CD40 ligand expression, interleukin-4 secretin and efficient help for antibody production by B-cells. Eur J Immunol 23:3120, 1993.

Delves PJ and Roitt IM. Idiotypic determinants on human thyroglobulin autoantibodies derived from the serum of Hashimoto patients and EB virus transformed cell lines. Clin Exp Immunol 57:33, 1984.

Deen C, Claassen E, Gerritse K, Zegers ND and Boersma W. An improved and modified carbodiimide coupling method for synthetic peptides: Enhanced antipeptide antibody responses. J Imm Meth 129:119, 1990.

De Simone R, Giampaolo A, Giometto B, Gallo P, Levi G, Peschle C and Aloisi F. The costimulatory molecule B7 is expressed on human microglia in culture and in multiple sclerosis acute lesions. J Neuropath Exp Neurology 54:157, 1995.

Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA and Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. Science 261:1328, 1993.

Durie FH, Foy TM, Masters SR, Laman JD and Noelle RJ. The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol Today 15:406, 1994.

Ebers G. A population based study of multiple sclerosis in twins. N Engl J Med 315:1638, 1986.

Eikelenboom P. Characterization of non-lymphoid cells in the white pulp of the mouse spleen: An *in vivo* and *in vitro* study. Cell Tiss Res 195:445, 1978.

Endo T, Stewart SS, Kundu SK, Osovitz S and Marcus DM. Antibodies to glycosphingolipids in patients with multiple sclerosis. Ann NY Acad Sc 436:213, 1984.

Endoh M, Rapoport SI and Tabira T. Studies of experimental allergic encephalomyelitis in old mice. J Neuroimmunol 29:21, 1990.

England MA and Wakely J. A colour atlas of the brain & spinal cord. An introduction to normal neuroanatomy. Wolfe Publishing Ltd., 1991.

Eylar EH. In: Rowland LP, Ed. Proceedings of the association for research in nervous and mental disease. Baltimore, The Williams & Wilkens Company, 49:55, 1971.

Faria AMC, Garcia G, Rios MJC, Michalaros CL and Vaz NM. Decrease in susceptibility to oral tolerance induction and occurrence of oral immunization to ovalbumin in 20-38 week old mice. The effect of interval between oral exposures and rate of antigen intake in the oral immunization. Immunology 78:147, 1993.

Felgenhauer K and Reiber H. The diagnostic significance of antibody specificity indices in multiple sclerosis and herpes virus induced diseases of the central nervous system. Clin Invest 70:28, 1992.

Fernandez CF, Shahani KM and Amer MA. Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy products. Fems Microbiol 46:343, 1987.

Forrest BD. Identification of an intestinal immune response using peripheral blood lymphocytes. Lancet 1:81, 1988.

Foy TM, Shepherd DM, Durie FH, Aruffo A, Ledbetter JA and Noelle RJ. *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. J Exp Med 178:1567, 1993.

Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E and Noelle RJ. GP39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J Exp Med 180:157, 1994.

Fuhrman JA and Cebra JJ. Special features of the priming process for a secretory IgA response. J Exp Med 153:534, 1981.

Fuji Y and Lindstrom J. Specificity of the T-cell immune response to acetylcholine receptor in experimental autoimmune Myasthenia Gravis response to subunits and synthetic peptides. J Immunol 140:1830, 1988.

Freedman MS and Antel JP. Immunoregulatory circuits in MS: Is there a "short"? Ann Neurol 24:183, 1988.

Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C and Fontana A. Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. Eur J Immunol 17:1271, 1987.

Fritz RB, Skeen MJ, Chou CHJ, Garcia M and Egorov K. Major histocompatibility complex-linked control of the murine immune response to myelin basic protein. J Immunol 134:2328, 1985.

Gall JC, Hayles AB, Siekert RG and Keith HM. Multiple sclerosis in children: A clinical study of 40 cases with onset in childhood. Pediatrics 21:703, 1958.

Geffard M, Boullerne A and Brochet B. Seric immune complexes in multiple sclerosis do not contain MBP epitopes. Brain Res Bull 30:365, 1993.

Gerritse K, Van Gorcom RFM, Fasbender MJ, Lange J, Schellekens MM, Zegers ND, Claassen E and Boersma WJA. Specific immuno-detection of benzoate-para-hydroxylase with antibodies raised against synthetic peptides. Biochem Biophys Res Commun 167:33, 1990a.

Gerritse K, Posno M, Schellekens MM, Boersma WJA and Claassen E. Oral administration of TNP-*Lactobacillus* conjugates in mice: A model for evaluation of mucosal- and systemic immune responses and memory formation elicited by transformed Lactobacilli. Res Microbiol 141:955, 1990b.

Gerritse K, Posno M, Schellekens MM, Boersma WJA and Claassen E. Mucosal immune responses and systemic immunological memory after oral administration of TNP-*Lactobacillus* conjugates in mice. In: Lymphatic Tissues and *in vivo* Immune Responses. Imhof B, Berrih-Aknin S and Ezine S, Eds. New York, Marcel Dekker Inc., 497, 1991a.

Gerritse K, Fasbender M, Boersma W and Claassen E. Conjugate formation in urea to couple insoluble peptides to alkaline phosphatase for ELISA and *in situ* detection of antibody forming cells. J Histochem Cytochem 39:987, 1991b.

Gerritse K, Slierendregt B, Deen C, Fasbender M, Boersma W and Claassen E. *In situ* detection of self reactive epitope specific antibody forming cells in the central nervous system of EAE rhesus monkey. EOS J of Immunol Immunopharmacol 13:63, 1993.

Gerritse K, Deen C, Fasbender M, Ravid R, Boersma W and Claassen E. The involvement of specific anti myelin basic protein antibody forming cells in multiple sclerosis immunopathology. J Neuroimmunol 49:153, 1994.

Ghezzi A, Manara F, Marforio S and Rocca M. Multiple sclerosis in childhood (a clinical and statistical study of 58 cases). Acta Neurol 33:157, 1978.

Goldblum RM, Ahlstedt S, Carlsson B, Hanson L-Å, Jodal U, Lidin-Janson G and Sohl-Åkerlund A. Antibody-forming cells in human colostrum after oral immunization. Nature 257:797, 1975.

Gorbach SL. Lactic acid bacteria and human health. Ann Medicine 22:37, 1990.

Górny MK, Wróblewska Z, Pleasure D, Miller SL, Wajgt A and Koprowski H. CSF antibodies to myelin basic protein and oligodendrocytes in multiple sclerosis and other neurological diseases. Acta Neurol Scand 67:338, 1983.

Goverman J, Woods A, Larson L, Weiner LP, Hood L and Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell 72:551, 1993.

Graaf de J, Van der Hoeven JH, Hoogstraten MC and Minderhoud JM. Multiple sclerose. Utrecht, Bohn, Scheltema & Holkema, 1988.

Greenstein JI, Knobler RL, Johnson KP, Marcus SG, Panitch HS, Lublin FD, Hassett RM, Wilson JR, Russick AM, Haley AS and Grant SV. A phase I clinic trial of human recombinant beta interferon in relapsing-remitting multiple sclerosis. J Neuroimmunol 16:66, 1987.

Groot de CJA, Sminia T, Dijkstra CD, Van de Pal RHM and Lopes-Cardozo M. Interferon-gamma induced Ia antigen expression on cultured neuroglial cells and brain macrophages from rat spinal cord and cerebrum. Intern J Neurosci 59:53, 1991.

Hafler DA, Fallis RJ, Dawson DM, Schlossman SF, Reinherz EL and Weiner HL. Immunological responses of progressive multiple sclerosis patients treated with an anti-T-cell monoclonal antibody, anti-T12. Neurology 36:777, 1986.

Hafler DA and Weiner HL. Immunosuppression with monoclonal antibodies in multiple sclerosis. Neurology 38:42, 1988.

Hafler DA, Orav J, Gertz R, Stazzone L and Weiner HL. Immunologic effects of cyclophosphamide/ACTH in patients with chronic progresssive multiple sclerosis. J Neuroimmunol 32:149, 1991.

Hale TL. Hybrid vaccines using *Escherichia coli* as an antigen carrier. Res Microbiol 141:913, 1990.

Hanson DG, Vaz NM, Rowlings LA and Lynch JM. Inhibtion of specific immune responses by feeding protein antigen. II. Effects on prior passive and active immunization. J Immunol 122:2261, 1979.

Hanson DG. Ontogeny of orally induced to soluble proteins in mice. I. Priming and tolerance in newborns. J Immunol 127:1518, 1981.

Hashim G, Vandenbark AA, Gold DP, Diamanduros T and Offner H. T-cell lines specific for an immunodominant epitope of human basic protein define an encephalitogenic determinant for experimental autoimmune encephalomyelitis-resistant LOU/M rats. J Immunol 146:515, 1991.

Hashim GA, Offner H, Wang RY, Shukla K, Carvalho E, Morrison WJ and Vandenbark AA. Spontaneous development of protective anti-T cell receptor autoimmunity targeted against a natural EAE-regulatory idiotope located within the 35-59 region of the TCR-Vβ8.2 chain. J Immunol 149:2803, 1992.

Hauser SL, Dawson DM, Lehrich JR, Beal MF, Kevy SV, Propper RD, Mills JA and Weiner HL. Intensive immunosuppression in progressive multiple sclerosis. A randomized, three-arm study of high-dose intravenous cyclophosphamide, plasma exchange, and ACTH. New Engl J Med 308:173, 1983.

Havekes LM, De Wit E, Leuven JG, Klasen E, Utermann G, Weber W and Beisiegel U. Apolipoprotein E3-Leiden: A new variant of human apolipoprotein E associated with familial type III hyperlipoproteinemia. Hum Genet 73:157, 1986.

Heckels JE, Virji M and Tinsley CR. Vaccination against gonorrhoea: The potential protective effect of immunization with a synthetic peptide containing a conserved epitope of gonococcal outer membrane protein IB. Vaccine 8:225, 1990.

Henneberg A, Mayle DM and Kornhuber HH. Antibodies to brain tissue in sera of patients with chronic progressive multiple sclerosis. J Neuroimmunol 34:223, 1991.

Heppell LMJ and Kilshaw PJ. Immune responses of guinea pigs to dietary protein. I. Induction of tolerance by feeding with ovalbumin. Int Arch Allergy Appl Immunol 68:54, 1982.

Hickey WF and Kimura H. Perivascular microglia cells of the CNS are bone marrow-derived and present antigen *in vivo*. Science 239:290, 1988.

Higgins PJ and Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. J Immunol 140:440, 1988.

Hirsch HE and Parks ME. Serological reactions against glycolipid-sensitized liposomes in multiple sclerosis. Nature 264:785, 1976.

Hoogstraten van IM, Andersen KE, Von Blomberg BM, Boden D, Bruynzeel DP, Burrows D, Camarasa JG, Dooms-Goossens A, Kraal G and Lahti A. Reduced frequency of nickel allergy upon oral nickel contact at an early age. Clin Exp Immunol 85:441, 1991.

Horikawa Y, Tsubaki T and Nakajima M. Rubella antibody in multiple sclerosis. Lancet 996, 1973.

Huitinga I, Van Rooijen N, De Groot CJA, Uitdehaag BMJ and Dijkstra CD. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J Exp Med 172:1025, 1990.

Huitinga I, Ruuls SR, Jung S, Hartung HP, Van Rooijen N and Dijkstra CD. The role of macrophages infiltrating the central nervous system of Lewis rats with T cell-induced experimental allergic encephalomyelitis. Huitinga I, Ph D Thesis, Febodruk, Enschede, 52, 1992.

Husband AJ and Gowans JL. The origin and antigen-dependent distribution of IgA-containing cells in the intestine. J Exp Med 148:1146, 1978.

Janssen PSL, Van Nispen JW, Melgers PATA, Van den Bogaart HWM, Hamelinck RLAE and Goverde BC. HPLC analysis of phenylthiocarbamyl (PTC) amino acids. I. Evaluation and optimization of the procedure. Chromatographia 22:351, 1986.

Jansson L, Olsson T, Höjeberg B and Holmdahl R. Chronic experimental autoimmune encephalomyelitis induced by the 89-101 myelin basic protein peptide in BALB/c RIII (H-2) mice. Eur J Immunology 216:93, 1991.

Jerne NK. Towards a network theory of the immune system. Ann Immunol 125:373, 1975.

Jeurissen SHM, Claassen E, Van Rooijen N and Kraal G. Intra-intestinal priming leads to antigen-specific IgA memory cells in peripheral lymphoid organs. Immunology 56:417, 1985.

Jingwu Z, Chin Y, Henderikx P, Medaer R, Chou CH and Raus JC. Antibodies to myelin basic protein and measles virus in multiple sclerosis: Precursor frequency analysis of the antibody producing B-cells. Autoimmunity 11:27, 1991.

Johnson D, Sato S, Quarles RH, Inuzuka T, Brady RO and Tourtelotte WW. Quantitation of the myelin-associated glycoprotein in human nervous tissue from controls and multiple sclerosis patients. J Neurochem 46:1086, 1986.

Jones LA, Chin LT, Longo DL and Kruisbeek AM. Peripheral clonal elimination of functional T cells. Science 250:1726, 1990.

Jones RE, Bourdette D, Offner H and Vandenbark AA. The synthetic peptide 87-99 peptide of myelin basic protein is encephalitogenic in Buffalo rats. J Neuroimmunol 37:203, 1992.

Kagnoff MF. Effects of antigen-feeding on intestinal and systemic immune responses. II. Suppression of delayed-type hypersensitivity reactions. J Immunol 120:1509, 1978.

Karkhanis YD, Carlo DJ, Brostoff SW and Eylar EH. Allergic encephalomyelitis. Isolation of an encephalitogenic peptide active in the monkey. J Biol Chem 5:1718, 1975.

Karpus WJ and Swanborg RH. Protection against experimental autoimmune encephalomyelitis requires both $CD4^+$ T suppressor cells and myelin basic protein primed B cells. J Neuroimmunol 33:173, 1991.

Karpus WJ, Gould KE and Swanborg RH. $CD4^+$ suppressor cells of autoimmune encephalomyelitis respond to T cell receptor-associated determinants on effector cells by interleukin-4 secretion. Eur J Immunol 22:1757, 1992.

Kato I, Yokokura T and Mutai M. Augmentation of mouse natural killer cell activity by *Lactobacillus casei* and its surface antigens. Microbiol Immunol 28:209, 1984.

Kerlero de Rosbo N and Bernard CCA. Multiple sclerosis brain immunoglobulins stimulate myelin basic protein degradation in human myelin. A new cause of demyelination. J Neurochem 53:513, 1989.

Kerlero de Rosbo N, Honegger P, Lassmann H and Matthieu JM. Demyelination induced in aggregating brain cell cultures by a monoclonal antibody against myelin-oligodendroglial glycoprotein (MOG). J Neurochem 55:583, 1990.

Khoury SJ, Lider O, Al-Sabbagh A and Weiner HL. Suppression of experimental autoimune encephalomyelitis by oral administration of myelin basic protein. Cell Immunol 131:302, 1990.

Khoury SJ, Hancock WW and Weiner HL. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. J Exp Med 176:1355, 1992.

Killen JA and Swanborg RH. Regulation of experimental allergic encephalomyelitis. IV. Further characterization of post recovery suppressor cells. J Neuroimmunol 3:159, 1982.

Kitagawa T and Aikawa T. Enzyme coupled immunoassay of insulin using a novel coupling agent. J Biochem 79:233, 1976.

Klasen EA, Rigutti A, Bos A and Bernini LF. Development of a screening system for detection of somatic mutations. I. Enzyme immunoassay for detection of antibodies against specific haemoglobin determinants. J Imm Meth 54:241, 1982.

Klasen EA, Rigutti A, Bos A and Bernini LF. Development of a screening system for detection of somatic mutations. II. The use of peptides and insoluble protein fragments in a non-competitive solid-phase enzyme immunoassay. J Imm Meth 59:281, 1983.

Kleeman EG and Klaenhammer TR. Adherence of *Lactobacillus* species to human fetal intestinal cells. J Dairy Sci 65:2063, 1982.

Knobler RL, Panitch HS, Braheny SL, Sipe JC, Rice GP, Huddlestone JR, Francis GS, Hooper CJ, Kamin Lewis RM and Johnson KP. Clinical trial of natural alpha interferon in multiple sclerosis. Ann NY Acad Sci 436:382, 1984.

Kono DH, Urban JL, Horvath SJ, Ando DG, Saavedra RA and Hood L. Two minor determinants of myelin basic protein induce experimental allergic encephalomyelitis in SJL/J mice. J Exp Med 168:213, 1988.

Kuchroo VK, Sobel RA, Yamamura T, Greenfield E, Dorf ME and Lees MB. Induction experimental allergic encephalomyelitis by myelin proteolipid-protein specific T-cell clones and synthetic peptides. Pathobiology 59:305, 1991.

Kurdi A, Ayesh I, Abdallat A, Maayta U, McDonald WI, Compston DAS and Batchelor JR. Different B lymphocyte alloantigens associated with multiple sclerosis in Arabs and Northern Europeans. Lancet 1:1123, 1977.

Kurtzke JF and Hyllested K. Multiple sclerosis in the Faroe Islands: I. Clinical and epidemiological features. Ann Neurol 5:6, 1979.

Kurtzke JF. The geographical distribution of multiple sclerosis: An update with special reference to Europe and the Mediterranean region. Acta Neurol Scand 62:65, 1980a.

Kurtzke JF, Gudmunsun KR and Bergmann S. Multiple sclerosis in Iceland: A post war epidemic. Neurology 30:437, 1980b.

Kurtzke JF. Rating neurological impairment in multiple sclerosis: An expanded disability status scale (EDSS). Neurology 33:1444, 1983a.

Kurtzke JF. Epidemiology of multiple sclerosis. In: Multiple Sclerosis. Pathology, diagnosis and management. Kurtzke JF, Adams CWM and Toutelotte WW, Eds. London, Chapman and Hall, 47, 1983b.

Lam DKC. The blood-central nervous system barrier in acute experimental allergic encephalomyelitis. In: The Blood-Brain barrier in Health and Disease. Suckling AJ, Rumsby MG and Bradbury MWB, Eds. Ellis Horwood Ltd., Chichester, UK, 158, 1986.

Laman JD, Claassen E, Van Rooijen N and Boersma WJA. Immune complexes on follicular dendritic cells as a target for cytolytic cells in AIDS. AIDS 3:543, 1989.

Laman JD, Gerritse K, Fasbender M, Boersma WJA, Van Rooijen N and Claassen E. Double immunocytochemical staining for the *in vivo* detection of epitope specificity and isotype of antibody forming cells against synthetic peptides homologous to human immunodeficiency virus-1. J Histochem Cytochem 38:457, 1990.

Laman JD, Rácz P, Tenner-Rácz K, Klasmeier M, Fasbender MJ, Neelen C, Zegers ND, Dietrich M, Boersma WJA and Claassen E. Immunocytochemical determination of antigen- and epitope-specificity of HIV-1 specific b-cells in lymph node biopsies from HIV-1 infected persons. AIDS 5:255, 1991a.

Laman JD, Van den Eertwegh AJM, Claassen E and Van Rooijen N. Cell-Cell interactions. *In situ* studies of splenic humoral immune responses. In: Immune Accessory Cells. CRC press, Fornusek L and Vetvicka V, Eds. Boca Raton, USA, 201, 1991b.

Laman JD, Van den Eertwegh AJM, Deen C, Vermeulen N, Boersma WJA and Claassen E. Synthetic peptide conjugates with horseradish peroxidase and ß-galactosidase for use in epitope-specific immunocytochemistry and ELISA. J Immunol Methods 145:1, 1991c.

Laman JD, Kors N, Heeney J, Boersma WJA and Claassen E. Fixation of cryosections under HIV-1 inactivating conditions: Integrity of antigen binding sites and cell surface antigens. Histochemistry 96:177, 1991d.

Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature 314:537, 1985.

Lanzavecchia A. Antigen uptake and accumulation in antigen-specific B cells. Immunol Rev 99:39, 1987.

Lassila O, Vainio O and Matzinger P. Can B cells turn on virgin T cells. Nature 334:253, 1988.

Lassmann H, Brunner C, Bradl M and Linington C. Experimental allergic encephalomyelitis: The balance between encephalitogenic T lymphocytes and demyelinating antibodies determines size and structure of demyelinated lesions. Acta Neuropathol 75:566, 1988.

Lebar R, Lubetzki C, Vincent C, Lombrail P and Boutry JM. The M2 autoantigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane. Clin Exp Immunol 66:423, 1986.

Leclerc C, Charbit A, Molla A and Hofnung M. Antibody response to a foreign epitope expressed at the surface of recombinant bacteria: Importance of the route of immunization. Vaccine 7:242, 1989.

Leer RJ, Van Luijk N, Posno M and Pouwels PH. Structural and functional analysis of two cryptic plasmids from *Lactobacillus pentosus* MD353 and *Lactobacillus plantarum* ATCC 8014. Molec Gen Gemet 234:265, 1992.

Leer RJ, Christiaens H, Peters L, Posno M and Pouwels PH. Gen-disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: Isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. Mol Gen Genet 239:269, 1993.

Lees MB and Brostoff SW. Proteins of myelin. In: Myelin. Morell P, Ed. New York, Plenum Publishing Corp., 197, 1984.

Lehman TJ, Hanson V, Zvaifler N, Sharp G and Alspaugh M. Antibodies to nonhistone nuclear antigens and antilymphocyte antibodies among children and adults with systemic lupus erythematosus and their relatives. J Rheumatol 11:644, 1984.

Lehmann D and Ben-Nun A. Bacterial agents protect against autoimmune disease. I. Mice pre-exposed to Bordetella pertussis or Mycobacterium tuberculosis are highly refractory to induction of experimental autoimmune encephalomyelitis. J Autoimmun 5:675, 1992.

Lencner A, Lencner H, Brilis V, Brilene T, Mikelsaar M and Turi M. The defense function of the digestive tract lactoflora. Nahrung 31:405, 1987.

Lernmark A, Dyrberg T, Terenius L and Hökfelt B. Molecular mimicry in health and disease. Int. Congress Series, Exerpta Medica, Amsterdam, 1988.

Levine S, Saltzman A and Deibler GE. Peptides of myelin basic protein are encephalitogenic in rats without the aid of emulsions. PSEBM 195:325, 1990.

Lider O, Santos LMB, Lee CSY, Higgins PJ and Weiner HL. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. II. Suppression of disease and *in vitro* immune responses is mediated by antigen-specific $CD8^+$ T lymphocytes. J Immunol 142:748, 1989.

Linington C, Bradl M, Lassmann H, Brunner C and Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol 130:443, 1988.

Linington C, Morgan BP, Scolding NJ, Wilkins P, Piddlesden S and Compston DAS. The role of complement in the pathogenesis of experimental allergic encephalomyelitis. Brain 112:895, 1990.

Link H, Baig S, Olsson O, Jiang YP, Höjeberg B and Olsson T. Persistent anti-myelin basic protein IgG antibody response in multiple sclerosis cerebrospinal fluid. J Neuroimmunol 28:237, 1990.

Liu Y-J, Oldfield S and MacLennan ICM. Memory B-cells in T-cell dependent antibody responses colonize the splenic marginal zones. Eur J Immunol 18:355, 1988.

Lokman BC, Van Santen P, Verdoes JC, Leer RJ, Posno M and Pouwels PH. Organisation and nucleotide sequence of three genes involved in D-xylose catabolism in *Lactobacillus pentosus*. Mol Gen Genet 230:161, 1991.

Low NL and Carter S. Multiple sclerosis in children. Pediatrics 18:24, 1956.

Lumsden CE. The neuropathology of multiple sclerosis. In: Handbook of clinical immunology. Vinken PJ, Bruyn GW, Eds. Amsterdam, North Holland Publishing Company, 9:217, 1970.

Lycke N and Holmgren J. Strong adjuvans properties of choleratoxin on gut mucosal immune responses to orally presented antigens. Immunology 59:301, 1986.

Lyman WD, Roth GA, Brosnan CF, Bornstein MB and Raine CS. Quantitation of antigen specific T-cell induced demyelination *in vitro*. J Neuroimmunol 17:175, 1988.

Macphee IAM, Day MJ and Mason DW. The role of serum factors in the suppression of experimental allergic encephalomyelitis: Evidence for immunoregulation by antibody to the encephalitogenic peptide. Immunology 70:527, 1990.

Martin R, Jaraquemada D, Flerlage M, Richert J, Whitaker J, Long EO, McFarlin DE and McFarland HF. Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T-cell lines from multiple sclerosis patients and healthy individuals. J Immunol 145:540, 1990.

Mason DY and Sammons R. Alkaline phospatase and peroxidase for double immunoenzymatic labeling of cellular constituents. J Clin Pathol 31:454, 1978.

Matthews WB. Clinical aspects. In: McAlpine's multiple sclerosis. Matthews WB, Ed., 2nd Ed. Longman Singapore Publishers Ltd., Singapore, 43, 1991.

McAlpine D, Lumsden CE and Acheson ED. Multiple sclerosis: A reappraisal Edinburgh: Churchill Livingstone, 1972.

McDonald WI and Halliday AM. Diagnosis and classification of multiple sclerosis. Br Med Bull 33:4, 1977.

McIntyre DA and Harlander SK. Genetic transformation of intact *Lactococcus lactis* subsp. *lactis* by high-voltage electroporation. Appl Environ Microbiol 55:604, 1989.

Melamed D and Friedman A. Modification of the immune response by oral tolerance: Antigen requirements and interaction with immunogenic stimuli. Cell Immunol 146:412, 1993.

Merrifield RB. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85:2149, 1963.

Mestecky J. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol 7:265, 1987.

Mestecky J and McGhee FR. Immunoglobulin A (IgA): Molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv Immunol 40:153, 1987.

Meyermann R, Lampert PW, Korr H and Wekerle H. The blood brain barrier. A strict border to lymphoid cells? In: Stroke and Microcirculation. Cervós-Navarro and Ferszt R, Eds. Raven Press, New York, 289, 1987.

Miller A, Lider O, Abramsky O and Weiner H. Orally administered myelin basic protein in neonates primes for immune responses and enhances experimental autoimmune encephalomyelitis in adult animals. Eur J Immunol 24:1026, 1994.

Miller SD and Hanson DG. Inhibition of specific immune responses by feeding protein antigens. IV. Evidence for tolerance and specific active suppression of cell-mediated immune responses to ovalbumin. J Immunol 123:2344, 1979.

Mishell BB, Shiigi SM, Henry C, Chan EL, North J, Gallily R, Slomich M, Miller K, Marbrook J, Parks D and Good AH. Preparation of mouse spleen cell suspensions. In: Selected methods in cellular Immunology. Mishell BB and Shiigi SM, Eds. Freeman and Company, 3, 1980.

Miyamoto H, Walker JE, Ginsburg AH, Burks J, McIntosh K and Kempe CH. Antibodies to vaccinic and measles virus in multiple sclerosis patients. Archives of Neurology 33:414, 1976.

Mohan C, Shi Y, Laman JD and Datta SK. Interaction between CD40 and its ligand gp39 in the development of murine lupus nephritis. J Immunol 154:1470, 1995.

Mokhtarian F, McFarlin DE and Raine CS. Adoptive transfer of myelin basic protein-sensitized T-cells produces chronic relapsing demyelinating disease in mice. Nature 309:356, 1984.

Moller JR, Yanagisawa K, Brady RO, Tourtelotte WW and Quarles RH. Myelin associated glycoprotein in multiple sclerosis lesions: A quantitative and qualitative analysis. Ann Neurol 22:469, 1987.

Moller JR, Johnson D, Brady RO, Tourtellotte WW and Quarles RH. Antibodies to myelin-associated glycoprotein (MAG) in the cerebrospinal fluid of multiple sclerosis patients. J Neuroimmunol 22:55, 1989.

Muriana PM and Klaenhammer TR. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus* 88. Appl Environ Microbiol 53:553, 1987.

Nagler-Anderson C, Bober LA, Robinson ME, Siskind GW and GJ Thorbecke. Suppression of type II collagen-induced arthritis by intragastric administration of soluble type II collagen. Proc Natl Acad Sci USA 83:7443, 1986.

Naiki M, Takeoka Y, Kurimoto Y, Matsuoka T, Suehiro S, Imai Y, Osawa T and Gershwin ME. Neurotropin inhibits experimental allergic encephalomyelitis (EAE) in Lewis rats. Int J Immunopharmacol 13:235, 1991.

Nakane PK. Simultanious localization of multiple tissue antigens using the peroxidase-labeled antibody method: A study on pituitary glands of the rat. J Histochem Cytochem 16:557, 1968.

Nanno M, Ohwaki M and Mutai M. Induction by *Lactobacillus casei* of increase in macrophage colony forming cells and serum colony stimulating activity in mice. Jpn J Cancer Res 77:703, 1986.

Nedrud JG, Liang X, Hague N and Lamm NE. Combined oral/nasal immunization protects mice from Sendai virus infection. J Immunol 139:3484, 1987.

Nes IF. Plasmid profiles of ten strains of *Lactobacillus plantarum*. FEMS Microbiol Lett 21:359, 1984.

Noelle RJ, Daum J, Bartlett WC, McCann J and Shepherd DM. Cognate interactions between helper T cells and B cells. V. Recognition of T helper function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. J Immunol 146:1118, 1991.

Noelle RJ, Roy M, Shepherd DM, Stamenkovic I, Ledbetter JA and Aruffo A. A 39-kDa protein on activated helper T-cells binds CD40 and transduces the signal for cognate activation of B-cells. Proc Natl Acad Sci USA 89:6550, 1992.

Norrby E. Viral antibodies and multiple sclerosis. Progr Med Virol 24:1, 1978.

Nussenblatt RB, Caspi RR, Mahdi R, Chan CC, Roberge F, Lier O and Weiner HL. Inhibition of S-antigen induced autoimmune uveoretinitis by oral indiction of tolerance with S-antigen. J Immunol 144:1689, 1990.

Offner H, Hashim GA and Vandenbark AA. Response of rat encephalitogenic T lymphocytes lines to a synthetic peptides of myelin basic protein. J Neurosci Res 17:344, 1987.

Offner H, Hashim GA, Celnik B, Galang A, Li XB, Burns FR, Shen N, Heber-Katz E and Vandenbark AA. T-cell determinants of myelin basic protein include a unique encephalitogenic I-E restricted epitope for Lewis rats. J Exp Med 170:355, 1989.

Offner H, Hashim GA and Vandenbark AA. T cell receptor peptide therapy triggers autoregulation of experimental encephalomyelitis. Science 251:430, 1990.

Ota K, Matsui M, Milford EL, Mackin GA, Weiner HL and Hafler DA. T-cell recognition of an immuno-dominant myelin basic protein epitope in multiple sclerosis. Nature 346:183, 1990.

Ormerod IEC, Miller DH, McDonald WI, Du Boulay EPGH, Rudge P, Kendall BE, Moseley IF, Johnson G, Tofts PS, Halliday AM, Bronstein AM, Scaravilli F, Harding AE, Barnes D and Zilkha KJ. The role of NMR imaging in the assessment of multiple sclerosis and isolated neurological lesions. A quantitative study. Brain 110:1579, 1987.

Oger J, Willenboughby E and Paty DW. Immune function in MS: Correlation with disease activity as revealed by MRI. J Neuroimmunol 16:134, 1987.

Ozawa K, Saida T, Saida K, Nishitani H and Kameyama M. *In vivo* CNS demyelination mediated by anti-galactocerebroside antibody. Acta Neuropathol 77:621, 1989.

Pabst H, Day NK, Gewurz H and Good RA. Prevention of experimental allergic encephalomyelitis with cobra venom factor. Proc Soc Exp Biol Med 136:555, 1971.

Panitch HS, Francis GS, Hooper CJ, Merigan TC and Johnston KP. Serial immunological studies in multiple sclerosis patients treated systemically with human alpha interferon. Ann Neurol 18:434, 1985.

Panitch HS, Hirsch RL, Haley AS and Johnson KP. Exacerbations of multiple sclerosis in patients treated with gamma interferon. Lancet 1:893, 1987.

Paterson PY. Experimental autoimmune (allergic) encephalomyelitis: Induction, pathogenesis, and suppression. In: Tekstbook of immunopathology. Miescher PA and Muller-Eberhard HJ, Eds. Gruene and Straton, New York, 1:179, 1976.

Paulie S, Rosen A, Ehlin-Hendriksson B, Braesch-Andersen S, Jakobson E, Koho H and Perlmann P. The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoroprotein involved in growth signal transduction. J Immunol 142:590, 1989.

Perdigón G, Nader de Macias ME, Alvarez S, Medici M, Oliver G and Pesce de Ruiz Holgado AA. Effect of a mixture of *Lactobacillus casei* and *Lactobacillus acidophilus* administered orally on the immune system in mice. J Food Protec 49:986, 1986a.

Perdigón G, Alvarez S, Nader de Macias ME, Margni RA, Oliver G and Pesce de Ruiz Holgado AA. Lactobacilli administered orally induce release of enzymes from peritoneal macrophages in mice. Milchwissenschaft 41:344, 1986b.

Perdigón G, Nader de Macias ME, Alvarez S, Oliver G and Pesce de Ruiz Holgado AA. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect Immun 53:404, 1986c.

Perdigón M, De Macias MEN, Alvarez S, Oliver G and De Ruiz Holgado AP. Systemic augmentation of the immune response in mice by feeding fermented milks with *Lactobacillus casei* and *Lactobacillus acidophilus*. Immunol 63:17, 1988.

Peri BA, Theodore CM, Losonsky GA, Fishaut JM, Rothberg RM and Ogra PL. Antibody content of rabbit milk and serum following inhalation or ingestion of respiratory syncitial virus and bovine serum albumin. Clin Exp Immunol 48:91, 1982.

Pettinelli CB and McFarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after *in vitro* activation of lymph node cells by myelin basic protein: Requirement of Lyt $1^{+},2^{-}$T lymphocytes. J Immunol 127:1420, 1981.

Pettinelli CB, Fritz RB, Chou CHJ and McFarlin DE. Encephalitogenic activity of guinea pig myelin basic protein in the SJL mouse. J Immunol 129:1209, 1982.

Pierce NF and Cray Jr WC. Determinants of the localisation magnitude and duration of a specific mucosal IgA plasma cell response in enterically immunized rats. J Immunol 128:1311, 1982.

Polman CH, Matthaei I, De Groot CJA, Koetsier JC, Sminia T and Dijkstra CD. Low-dose cyclosporine A induces relapsing remitting experimental allergic encephalomyelitis in the Lewis rat. J Neuroimmunol 17:209, 1988.

Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, Johnson KP, Sibley WA, Silberberg DH and Tourtellotte WW. New diagnostic criteria for multiple sclerosis: Guidelines for research protocols. Ann Neurol 13:227, 1983.

Posno M, Leer RJ, Van Rijn JMM, Lokman BC and Pouwels PH. Transformation of *Lactobacillus plantarum* by plasmid DNA. In: Genetics and Biotechnology of bacilli. Ganesan AT and Hoch JA, Eds. Academic Press Inc., 2:397, 1988.

Posno M, Leer RJ, Lokman BC, Van Luijk N, Van Giezen MJF and Pouwels PH. Development of a host-vector system for *Lactobacillus*. Analysis of the stability of *Lactobacillus* plasmid DNA vectors. Proc Eur Congr Biotechnol, Copenhagen, Munksgaard, 900, 1990.

Posno M, Leer RJ, Van Luijk N, Van Giezen MJF, Heuvelmans PTHM, Lokman BC and Pouwels PH. Incompatibility of *Lactobacillus* vectors with replicons derived from small cryptic *Lactobacillus* plasmids and segregational instability of the introduced vectors. Appl Env Microbiol 57:1822, 1991a.

Posno M, Heuvelmans PTHM, Van Giezen MJF, Leer RJ and Pouwels PH. Complementation of the inability of *Lactobacillus* strains to utilize D-xylose with D-xylose catabolism-encoding genes of *Lactobacillus pentosus*. Appl Env Microbiol 57:2764, 1991b.

Pouwels PH, Leer RJ and Posno M. Genetic modification of *Lactobacillus*: A new approach towards strain improvement. In: Proc Symposium Actes du Colloque Lactic 91:133, 1992.

Pouwels PH and Leer RJ. Genetics of lactobacilli. Plasmids and gene expression. Antonie van Leeuwenhoek 64:85, 1993.

Pratt VM, Kiefer JR, Lahdetie J, Schleutket J, Hodes ME and Dlouhy SR. A new mutation in the proteolipid protein (PLP) gene in a German family with Pelizaeus-Merzbacher disease. Am J Med Genet 38:136, 1991.

Prineas JW and Connell F. Remyelination in multiple sclerosis. Ann Neurol 5:22, 1979.

Prineas JW. The neuropathology of multiple sclerosis. In: Handbook of clinical neurology, Demyelinating diseases. Koetsier JC, Ed. Amsterdam, Elsevier Science Publishers, 47:213, 1985.

Quarles RH, Everly JL and Brady RO. Evidence for the close association of a glycoprotein with myelin in rat brain. J Neurochem 21:1177, 1973.

Raine CS and Stone SH. Animal model for multiple sclerosis - chronic experimental allergic encephalomyelitis in inbred guinea pigs. NY State J Med 77:1693, 1977.

Raine CS. Experimental allergic encephalomyelitis and experimental allergic neuritis. In: Handbook of clinical neurology. Demyelinating diseases. Vinken PJ, Bruyn GW and Klawans HL, Eds. Rev Ser 3. Koetsier JC, Ed. Demyelinating diseases. Elsevier, Amsterdam, 3:429, 1985.

Raine CS, Lee SC, Scheinberg LC, Duijvestein AM and Cross AH. Adhesion molecules on endothelial cells in the central nervous system: An emerging area in the neuroimmunology of multiple sclerosis. Clin Immunol Immunopathol 57:173, 1990.

Rice GPA, Woelfel EL, Talbot PJ, Braheny SL, Sipe JC, Knobler RL, Merigan TC and Oldstone MB. Immunological complications in multiple sclerosis patients receiving interferon. Ann Neurol 18:439, 1985.

Rios MJC, Pereira MAC, Lopes LM, Faria AMC, Gontijo CM, Castanheira EB and Vaz NM. Tolerance induction and immunological priming initiated by mucosal contacts with protein antigens in inbred strains of mice. Braz J Med Biol Res 21:825, 1988.

Risau W, Engelhardt B and Wekerle H. Immune function of the blood brain barrier: Incomplete presentation of protein (auto-) antigens by rat brain microvascular endothelium *in vitro*. J Cell Biol 110:1757, 1990.

Robbins DS, Shirazi SY, Drysdale BE, Liebermann A and Shin HS. Production of cytotoxic factor for oligodendrocytes by stimulated astrocytes. J Immunol 139:2593, 1987.

Ron MA and Feinstein A. Multiple sclerosis and the mind. J Neurol Neurosurg Psychiatry 55:1, 1992.

Roux ME, McWilliams M, Phillips-Quagliata JM and Lamm ME. Differentiation pathway of Peyer's patches precursors of IgA plasma cells in the secretory immune system. Cell Immunol 61:141, 1981.

Ruijs TC, Olivier A and Antel JP. Serum cytotoxicity to human and rat oligodendrocytes in culture. Brain Res 517:99, 1990.

Sachs L. Applied statistics: A handbook of techniques. 2nd Ed. Heidelberg, Springer Verlag, 1984.

Saida T, Saida K and Silverberg DH. Demyelination produced by experimental allergic neuritis serum and anti-galactocerebroside antiserum in central nervous system cultures. Acta neuropathol 48:19, 1979.

Saklayen MG, Pesce AJ, Polak VE and Michael JG. Kinetics of oral tolerance: Study of variables affecting tolerance induced by oral administration of antigen. Int Arch Allergy Appl Immunol 73:5, 1984.

Salmi A, Gollmar Y, Norrby E and Panelius M. Antibodies against three different structural components of measles in patients with multiple sclerosis, their sibling and matched controls. Acta Pathol Microbiol Scan 81:627, 1973.

Sato K, Saito H, Tomioka H and Yokokura T. Enhancement of host resistance against Listeria infection by *Lactobacillus casei*: Efficacy of cell wall preparation of *Lactobacillus casei*. Microbiol Immunol 32:1189, 1988.

Satoh J, Kim SU and Kastrukoff LF. Cytolysis of oligodendrocytes is mediated by killer (K) cells but not by natural killer (NK) cells. J Neuroimmunol 31:199, 1991.

Savage DC. Microbial ecology of the gastrointestinal tract. Ann Rev Microbiol 31:107, 1977.

Savage DC. Morphological diversity among members of the gastrointestinal microflora. Int Rev Cytology 82:305, 1983.

Schumacher GA, Beebe G, Kibler RF, Kurland LT, Kurtzke JF, McDowell F, Nagler B, Sibley WA, Tourtellotte WW and Willmon TL. Problems of experimental trials of therapy in multiple sclerosis: Report by the panel of evaluation of experimental trials of therapy in multiple sclerosis. Ann NY Acad Sci 122:552, 1965.

Shively JE. Reversed-phase HPLC isolation and microsequence analysis. In: Methods of Protein Microcharacterization. The Humana Press Inc., 2:41, 1986.

Shu U, Kiniwa M, Wu CW, Maliszewski C, Vezzio N, Hakimi J, Gately M and Delespesse G. Activated T-cells induce interleukin-12 production by monocytes via CD40-CD40-ligand interaction. Eur J Immunol 25:1125, 1995.

Sjovall P and Christensen OB. Oral hyposensitization in allergic contact dermatitis. Semin Dermatol 9:206, 1991.

Sobel RA, Tuohy VK, Lu Z, Laursen RA and Lees MB. Acute experimental allergic encephalomyelitis in SJL/J mice induced by a synthetic peptide of myelin proteolipid protein. J Neuropathol Exp Neurol 49:468, 1990.

Sobel RA, Tuohy VK and Lees MB. Parental MHC molecule haplotype expression in SJL/J x SWR)F1 mice with acute experimental allergic encephalomyelitis induced with two different synthetic peptides of myelin proteolipid protein. J Immunology 146:543, 1991.

Springer TA, Dustin ML, Kishimoto TK and Marlin SD. The lymphocyte function associated LFA1, CD2, and LFA3 molecules: Cell adhesion receptors of the immune system. Ann Rev Immunol 5:223, 1987.

Stabel TJ, Mayfield JE, Tabatabai LB and Wannemuehler MJ. Swine immunity to an attenuated Salmonella typhimurium mutant containing a recombinant plasmid which codes for production of a 31-kilodalton protein of *Brucella abortus*. Infect Immun 59:2941, 1991.

Sternberger LA, Hardy PH, Cuculis JJ and Meyer HG. The unlabelled antibody enzyme method of immunohistochemistry: Preparation, and properties of soluble antigen-antibody complex (horseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315, 1970.

Stokes CR, Swarbrick ET and Soothill JF. Genetic differences in immune exclusion and partial tolerance to ingested antigens. Clin Exp Immunol 52:678, 1983.

Strober W, Richman LK and Elson CO. The regulation of gastrointestinal immune responses. Immunol Today 156, 1981.

Su XM and Sriram S. Treatment of chronic relapsing experimental allergic encephalomyelitis with the intravenous administration of splenocytes coupled to encephalitogenic peptide 91-103 of myelin basic protein. J Neuroimmunol 34:181, 1991.

Su XM and Sriram S. Analysis of TCR Vβ gene usage and encephalitogenicity of myelin basic protein peptide p91-103 reactive T-cell clones in SJL mice: Lack of evidence for V gene hypothesis. Cellular Immunology 141:485, 1992.

Suckling AJ, Pathak S, Jagelman S and Webb HE. Virus associated demyelination. A model using avirulent Semliki Forest virus infectionof mice. J Neurological Sci 36:147, 1978.

Sun D and Wekerle H. Ia-restricted encephalitogenic T-lymphocytes mediating EAE lyse autoantigen presenting astrocytes. Nature 320:70, 1986.

Sun JB, Olsson T, Wang WZ, Xiao BG, Kostulas V, Frederikson S, Ekre HP and Link H. Autoreactive T- and B-cells responding to myelin proteolipid protein in multiple sclerosis and controls. Eur J Immunol 21:1461, 1991.

Swanborg RH. Autoimmune effector cells. V. A monoclonal antibody specific for rat helper T lymphocytes inhibits adoptive transfer of autoimmune encephalomyelitis. J Immunol 130:1503, 1983.

Swank RL. Multiple sclerosis: Fat-oil relationship. Nutrition 7:368, 1991.

Tan P, Anasetti C, Hansen JA, Melrose J, Brunvand M, Bradshaw J, Ledbetter JA and Lindsley PS. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. J Exp Med 177:165, 1993.

Tannock GW, Dashkevicz MP and Feighner SD. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl Environ Microbiol 55:1848, 1989.

Thompson AJ, Kermode AG and Wicks D. Major differences in the dynamics of primary and secondary progressive multiple sclerosis. Am Neurol 29:53, 1991.

Thompson HSG and Staines NA. Could specific oral tolerance be a therapy for autoimmune disease? Immunol Today 11:396, 1990.

Tian L, Noelle RJ and Lawrence DA. Activated T cells enhance nitric oxide production by murine splenic macrophages through gp39 and LFA-1. Eur J Immuno 25:306, 1995.

Tourtelotte WW and Baumhefner RW. Comprehensive managment of multiple sclerosis. In: Multiple Sclerosis. Hallpike JF, Adams CWM and Tourtelotte WW, Eds. London, Chapman & Hall, 513, 1983.

Traugott U, Scheinberg LC and Raine CS. Multiple Sclerosis: Heterology among early T cells and Tg cells. Ann Neurol 11:182, 1982.

Traugott U and Raine CS. T- and B-cell distribution in multiple sclerosis (MS) lesions. J Neuropathol Exp Neurol 42:425, 1982.

Traugott U, Reinherz EL and Raine CS. Multiple Sclerosis. Distribution of T-cells, T-cell subsets and Ia-positive macrophages in lesions of different ages. J Neuroimmunol 4:201, 1983.

Trotter JL, Hickey WF, Van der Veen RC and Sulze L. Peripheral blood mononuclear cells from multiple sclerosis patients recognize myelin proteolipid protein and selected peptides. J Neuroimmunology 33:55, 1991.

Tuohy VK, Lu Z, Sobel RA, Laursen RA and Lees MB. A synthetic peptide from myelin proteolipid protein induces experimental allergic encephalomyelitis. J Immunol 141:1226, 1988.

Tuohy VK, Lu Z, Sobel RA, Laursen RA and Lees MB. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. J Immunol 142:1523, 1989.

Uhlenbrock D, Seidel D, Gehlen W, Beyer HK, Haan J, Dickmann E, Zeit T and Herbe E. MR imaging in multiple sclerosis: Comparison with clinical, CSF, and visual evoked potential findings. AJNR Am J Neuroradiol 9:59, 1988.

Ulvestad E, Williams K, Bjerkvig R, Tiekotter K, Antel J and Matre R. Human microglial cells have phenotypic and functional characteristics in common with both macrophages and dendritic antigen-presenting cells. J Leukoc Biol 56:732, 1994.

Vandenbark AA, Bourdette DN, Whitham R, Chou YK, Hashim GA and Offner H. T-cell receptor peptide therapy in EAE and MS. Clin and Exp Rheumatol 11:S51, 1993.

Van Denderen J, Hermans A, Meeuwsen T, Troelstra C, Zegers N, Boersma W, Grosveld G and Van Ewijk W. Antibody recognition of the tumor-specific bcr-abl joining region in chronic myeloid leukaemia. J Exp Med 169:87, 1989.

Van den Eertwegh AJM, Fasbender MJ, Schellekens MM, Van Oudenaren A, Boersma WJA and Claassen E. *In vivo* characterization of IFN-γ producing cells during a thymus independent immune response. J Immunol 177:439, 1991a.

Van den Eertwegh AJM and Claassen E. T-cells in the spleen: Localisation, cytokine production and cell-cell interactions. 38th Forum in Immunology. Research in Immunology 142:321, 1991b.

Van den Eertwegh AJM, Noelle RJ, Roy M, Shepherd DM, Aruffo A, Ledbetter JA, Boersma WJA and Claassen E. *In vivo* CD40-gp39 interactions are essential for thymus dependent humoral immunity. I. *In vivo* expression of CD40 ligand, cytokines and antibody production delineates sites of cognate T-B cell interactions. J Exp Med 178:1555, 1993.

Van den Eertwegh AJM, Van Ommen R, Noelle RJ, Savelkoul HFJ, Deen C, Boersma WJA and Claassen E. *In vivo* antigen localization, gp39 expression, cytokine production and antibody-formation are sequential events after injection of anti-mouse IgD antibodies. Submitted. 1994.

Van der Brugge-Gamelkoorn GJ, Claassen E and Sminia T. Anti-TNP-forming cells in bronchus-associated lymphoid tissue (BALT) and paratracheal lymph node (PTLN) of the rat after intratracheal priming and boosting with TNP-KLH. Immunology 54:405, 1986.

Van de Verg L, Herrington DA, Murphy JR, Wasserman SS, Formal SB and Levine MM. Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with bivalent *Salmonella typhi-Shigella sonnei* vaccine or infection by pathogenic *S. sonnei*. Infect Immun 58:2002, 1990.

Van Lambalgen R and Jonker M. Experimental allergic encephalomyelitis in rhesus monkeys: I. Immunological parameters in EAE resistant and susceptible rhesus monkeys. Clin Exp Immunol 68:100, 1987.

Van Noort JM and Van der Drift ACM. The selectivity of cathepsin D suggests an involvement of the enzyme in the generation of T-cell epitopes. J Biol Chem 264:14159, 1989.

Van Rooijen N. The limitations of immunoenzyme approaches to distinguish between <specific> and <non-specific> antibody forming cells, with particular respect to immunocytochemical studies on the *in situ* immune response. Histochem J 19:369, 1987.

Van Rooijen N, Kors N, Claassen E and Boorsma DM. Binding of different antigen-enzyme and antibody-enzyme conjugates by intracellular antibodies in cytoplasm and Golgi complex of plasma cells. A double immunocytochemical study. Histochemistry 83:61, 1985.

Van Rooijen N and Claassen E. Recent advances in the detection and characterization of specific antibody forming cells in tissue sections. A review. Histochemical J 18:465, 1986.

Van Rooijen N, Claassen E, Kraal G and Dijkstra CD. Cytological basis of immune functions of the spleen. Progress in Histochem Cytochem, 19:1, 1989.

Vass K and Lassmann H. Intrathecal application of interferon-gamma: Progressive appearance of MHC antigens within the rat nervous system. Am J Pathol 137:789, 1990.

Vaz NM, Maia LCS, Hanson DG and Lynch JM. Inhibition of homocytotropic antibody responses in adult mice by previous feeding with the specific antigen. J Allergy Clin Immunol 60:110, 1977.

Veen van der RC, Sobel RA and Lees MB. Chronic experimental allergic encephalomyelitis and antibody responses in rabbits immunized with bovine proteolipid apoprotein. J Neuroimmunol 11:321, 1986.

Veen van der RC, Trotter JL, Clark HB and Kapp JA. The adoptive transfer of chronic relapsing experimental allergic encephalomyelitis with lymph node cells sensitized to myelin proteolipid protein. J Neuroimmunol 21:183, 1989.

Vermeesch MK, Knapp PE, Skoff RP, Studzinski DM and Benjamins JA. Death of indvidual oligodendrocytes in jimpy brain precedes expression of proteolipid protein. Dev Neurosci 12:303, 1990.

Vescovo M, Bottazzi V, Sarra PG and Dellaglio F. Evidence of plasmid deoxyribonucleic acid in *Lactobacillus*. Microbiologica 4:413, 1981.

Voorbij HAM. Dendritic cells and the development of thyroid autoimmune disease and type I diabetes mellitus. Thesis Vrije Universiteit Amsterdam, Thesis Publishers Amsterdam, 1989.

Vos Q, Claassen E and Benner R. Enumeration of anti-insulin antibody-secreting cells using an improved spot-elisa. J Immunol Meth 126:89, 1990.

Wagner DH, Stout RD and Suttles J. Role of the CD40-CD40 ligand interaction in $CD4^+$ T cell contact-dependent activation of monocyte interleukin-1 synthesis. Eur J Immunol 24:3148, 1994.

Wahdan MH, Serie C, Cerisier Y, Sallam S, and Germanier R. A controlled field trial of live *Salmonella typhi* strain Ty 21a oral vacccine against typhoid: Three-year results. J Infect Dis 145:292, 1982.

Wajgt A and Gorny M. CSF antibodies to myelin basic protein and to myelin associated glycoprotein in multiple sclerosis. Evidence for intrathecal production of antibodies. Acta Neurol Scand 67:338, 1983.

Walsh MJ and Tourtelotte WW. In: Multiple Sclerosis. Hallpike JF, Adams CWM and Tourtelotte WW, Eds. London, Chapman & Hall, 275, 1983.

Wannemuhler MJ, Kiyono H, Babb JL, Michalek SM and McGhee JR. Lipopolysacchharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. J Immunol 129:969, 1982.

Warren KG and Catz I. Diagnostic value of cerebrospinal fluid anti-myelin basic protein in patients with multiple sclerosis. Ann Neurol 20:20, 1986.

Warren KG and Catz I. A myelin basic protein antibody cascade in purified IgG from cerebrospinal fluid of multiple sclerosis patients. J Neurol Sci 96:19, 1990.

Warren KG and Catz I. Synthetic peptide specificity of anti-myelin basic protein from multiple sclerosis cerebrospinal fluid. J Neuroimmunol 39:81, 1992a.

Warren KG and Catz I. Purification of primary antibodies of the myelin basic protein antibody cascade from multiple sclerosis patients. Immunoreactivity studies with homologous and heterologous antigens. Clin Invest Med 15:18, 1992b.

Warren KG and Catz I. Autoantibodies to myelin basic protein within multiple sclerosis central nervous system tissue. J Neurological Sci 115:169, 1993.

Warren KG, Catz I, Johnson E and Mielke B. Anti-myelin basic protein and anti-proteolipid protein specific forms of multiple sclerosis. Ann Neurol 35:280, 1994.

Webb HE and Fazakerley JK. Can viral envelope glycolipids produce auto-immunity, with reference to the CNS and multiple sclerosis? Neuropathol and Applied Neurobiol 10:1, 1984.

Weiner HL, Hauser SL, Hafler DA, Fallis RJ, Lehrich JR and Dawson DM. The use of cyclophosphamide in the treatment of multiple sclerosis. Ann NY Acad Sci 436:373, 1985.

Weiner HL, Zhang ZJ, Khoury SJ, Miller A, Al-Sabbagh A, Brod SA, Lider O, Higgins P, Sobel R, Nussenblatt RB and Hafler DA. Antigen-driven peripheral immune tolerance. Suppression of organ-specific autoimmune diseases by oral administration of autoantigens. Ann NY Acad Sci 636:227, 1991.

Weiner HL, Miller A, Zhang ZJ, Al-Sabbagh A, Matsui M, Khoury SJ, Nussenblatt R, Eisenbarth G and Hafler DA. Suppression of experimental autoimmune diseases via the oral administration of autoantigens. In: Advances in mucosal immunology, Adv Exp Med and Biology. Brandtzaeg, McGhee, Mesteckly, Sterzl and Tlaskalova, Eds. Plenum press, New York, 1992.

Weiner HL, Mackin GA, Matsui M, Orav EJ, Khoury SJ, Dawson DM and Hafler DA. Double blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. Science 259:1321, 1993.

Weiner LP. Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). Arch Neurol 28:298, 1973.

Weller PO. Pathology of multiple sclerosis. In: Multiple Sclerosis. Mathews WB, Acheson ED, Batchelor JR and Weller PO, Eds. McAlpine's Edinburgh Churchill Livingstone, 301, 1985.

Whitacre CC, Gienapp IE, Cox KL and Orsoz CG. FASEB J 3:6600, 1989.

Whitacre CC, Gienapp IE, Zhang X and Heber-Katz. FASEB J 4:949, 1990.

Whitacre CC, Gienapp IE, Orosz CG and Bitar DM. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. J Immunol 147:2155, 1991.

Whitaker JN. Myelin encephalitogenic protein fragments in cerebrospinal fluid of persons with multiple sclerosis. Neurology 27:911, 1977.

Whitaker JN. The presence of immunoreactive myelin basic protein peptide in urine of persons with multiple sclerosis. Ann neurol 22:648, 1987.

Whitham RH, Jones RE, Hashim GA, Hoy CM and Wang RY, Vandenbark AA and Offner H. Location of new encephalitogenic epitope (residues 43 to 64) in proteolipid protein that induces relapsing experimental autoimmune encephalomyelitis in PL/J and (SJL x PL) F1 mice. J Immunol 147:3803, 1991a.

Whitham RH, Bourdette DN, Hashim GA, Herndon RM, Ilg RC, Vandenbark AA and Offner H. Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. J Immunol 146:101, 1991b.

Willenborg DO. Mechanisms of recovery and acquired resistance in Lewis rat experimental allergic encephalomyelitis. Aust J Exp Biol Med Sci 59:125, 1981.

Willenborg DO and Prowse SJ. Immunoglobulin-deficient rats fail to develop experimental allergic encephalomyelitis. J Neuroimmunol 5:99, 1983.

Willenborg DO, Sjollema P and Danta G. Immunoglobulin deficient rats as donors and recipients of effector cells of EAE. J Neuroimmunol 11:93, 1986.

Williams RM, Krakowka S and Koestner A. *In vivo* demyelination by anti myelin antibodies. Acta Neuropathol 50:1, 1980.

Willoughby EW, Grochowski E, Li D, Oger J, Kastrukoff LF and Paty DW. Serial magnetic resonance scanning in multiple sclerosis: A second prospective study in relapsing patients. Ann Neurol 25:43, 1989.

Wold AE, Dahlgren UIH, Hanson LA, Mattsby Baltzer I and Midvetdt T. Differences between bacterial and food antigens in mucosal immunogenicity. Infect Immun 57:2666, 1989.

Wolf JL and Bye WA. The membranious epithelial cell (M) cell and the mucosal immune system. Ann Rev Med 35:95, 1984.

Wucherpfennig KW, Ota K, Endo N, Seidman JG, Rosenzweig A, Weiner HL and Hafler DA. Shared human T cell receptor Vβ usage to immunodominant regions of myelin basic protein. Science 248:1016, 1990.

Wucherpfennig KW, Weiner HL and Hafler DA. T-cell recognition of myelin basic protein. Immunol Today 12:277, 1991.

Xiao BG, Linington C and Link H. Antibodies to myelin oligodendrocyte glycoprotein in cerebrospinal fluid from patients with multiple sclerosis and controls. J Neuroimmunol 31:91, 1991.

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

Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L and Karin N. Prevention of experimental autoimmune encephalomyelitis by antibodies against $\alpha 4\beta 1$ integrin. Nature 356:63, 1992.

Yokokura T, Nomoto K, Shimizu T and Nomoto K. Enhancement of hematopoetic response of mice by subcutaneous administration of *Lactobacillus casei.* Infect Immun 52:156, 1986.

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

Young IR, Hall AS, Pallis CA, Legg NJ, Bydder GM and Steiner RE. Nuclear magnetic resonance imaging of the brain in multiple sclerosis. Lancet 2:1063, 1981.

Zamvill SS, Mitchell DJ, More AC, Kitamura K, Steinman L and Rothbard JB. T-cell epitope of the autoantigen myelin basic protein that includes encephalomyelitis. Nature 324:258, 1986.

Zamvill SS, Mitchell DJ, Powell MB, Sakai K, Rothbard JB and Steinman L. Multiple discrete encephalitogenic epitopes of the autoantigen myelin basic protein include a determinant for I-E class II restricted T-cells. J Exp Med 168:1181, 1988.

Zamvill SS and Steinmann L. The T lymphocyte in experimental allergic encephalomyelitis. Ann Rev Immunol 8:579, 1990.

Zanetta JP, Warter JM, Lehmann S, Tranchant C, Kuchler S and Vincendon G. Presence of antibodies to lectin CSL in the blood of patients with multiple sclerosis. CR Acad Sci III 311:327, 1990a.

Zanetta JP, Warter JM, Kuchler S, Marschal P, Rumbach L, Lehman S, Tranchant C, Reeber A and Vincendon G. Antibodies to cerebellar soluble lectin CSL in multiple sclerosis. The Lancet 335:1482, 1990b.

Zegers N, Gerritse K, Deen C, Boersma W and Claassen E. An improved conjugation method for controlled covalent coupling of synthetic peptides to proteins using glutaraldehyde in a dialysis method. J Immunol Meth 130:195, 1990.

Zegers N, Claassen E, Neelen C, Mulder E, Van Laar JH, Voorhorst MM, Berrevoets CA, Brinkmann AO, Van der Kwast TH, Ruizeveld de Winter JA, Trapman J and Boersma WJA. Epitope prediction and conformation for the human androgen receptor: Generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. Biochem Biophys Acta 1073:23, 1991.

Zhang JW, Chou CH, Hashim G, Medaer R and Raus JC. Preferential peptide specificity and HLA restriction of myelin basic protein-specific T-cell clones derived from MS patients. Cell Immunol 129:189, 1990.

SUMMARY

Multiple sclerosis (MS) is a chronic disease which affects the central nervous system (CNS) and is characterized by demyelinated areas and perivascular accumulation of inflammatory cells. Despite intensive scientific research both etiology and pathology of the disease remains largely unknown (*chapter 1*). Due to the complexity of clinical characteristics it is even very difficult to diagnose MS. Since several immune abnormalities are related to the diseases it is generally accepted that MS is the result of (auto)immune mediated demyelination.

One of the hallmarks of MS is an oligoclonal immunoglobulin banding pattern after isoelectric focusing of the cerebrospinal fluid. However, the role of humoral immunity in MS is as yet not clearly defined (*chapter 2.1*). To investigate the involvement of autoantigen specific immunoglobulin producing cells in the pathology of MS we have started a study to detect antigen specific B-cells in CNS tissues of MS patients. The detection of autoantigen specific B-cells and the *in situ* localization of these cells simultaneously can be accomplished by the use of immune-enzyme cytochemical staining techniques only. In *chapter 2.2* we describe the epitope specific detection of antibody forming cells (AFCs) in various tissues based on the application of antigen-enzyme conjugates. Using antigen-enzyme conjugates rare AFCs can easily be detected and enumerated. Even multiple antibody specificities can be visualized in a single tissue sample and tissue compartments. This approach allows careful localization and investigation of compartimentation of AFC subpopulations in human lymphoid and central nervous system tissues in order to investigate how B-cells may contribute to the pathogenesis of autoimmunity in man.

The detection of AFCs requires the construction of antigen-enzyme conjugates. Prior to, during and after the conjugation procedure it is necessary that both antigen, enzyme and antigen-enzyme conjugate are dissolvable in the media applied for the coupling chemistry. Most conjugation procedures are performed in aqueous buffers, which have no or minor influence on the three dimensional structure of the antigen or on the biological activity of the enzyme. Selected peptide sequences, representing specific epitopes, due to their hydrofobicity, not always readily dissolve in aqueous buffers. Therefore we have developed a new coupling method (*chapter 2.3*), which allows the conjugation of peptides, which have only very low solubility in aqueous buffers.

The structural protein components of the myelin sheaths, surrounding the neuronal axons, are considered to be putative target antigens for immune competent cells, which play a role in the pathogenesis of MS or experimental allergic encephalomyelitis (EAE). Whether or not AFCs directed against these myelin protein components play a role in the pathogenesis of MS or EAE is unclear, because the experiments performed sofar concerned the detection of AFCs and antibodies (Abs) in the sera and/or cerebrospinal fluid only. The use of myelin protein component-enzyme conjugates allows the detection of anti-myelin protein

component AFCs locally in the tissues of the CNS. In *chapter 2.4* we describe the construction and validation of a conjugate of horseradish peroxidase and myelin basic protein (MBP). MBP is one of the major protein components of CNS myelin. The MBP-enzyme conjugates were used to determine the presence of the anti-MBP AFCs in the spleens of mice and cerebrum of rhesus monkeys after induction of EAE. To investigate the putative role of anti-MBP AFCs in the pathogenesis of MS, we have used these conjugates to detect and localize anti-MBP AFCs in a panel of CNS tissues of MS patients, patients with other neurological diseases and healthy controls (*chapter 2.5*). The established presence of such cells is highly suggestive for a role of autoantigen reactive Abs in immunopathology of MS.

Abs and antibody producing B-cells specific for a variety of putative CNS antigens have been detected in both cerebrospinal fluid and sera of MS patients and in CNS tissue sections of MS patients as well. However, it is not clear whether the antigen specific B-cells, present within the CNS tissues of MS patient, will be activated by T-cells in the demyelinated areas of the CNS or in peripheral localized tissues. Multiple activation signals, surface protein interactions and co-stimulatory factors are required for the optimal proliferation and differentiation of autoantigen specific B-cells. Binding of activation T-cell membrane marker gp39 to its B-cell receptor CD40 is one of the requirements for the initiation of activation. In order to investigate a possible B-cell activation within CNS tissues of MS patients, CNS tissue sections were evaluated with respect to the presence of the gp39 activation marker on T-cells. In the experiments described in *chapter 3*, we have detected gp39 positive T-cells in the same affected CNS tissue areas of MS patients as were we have detected the MBP specific AFCs. This result indicate that autoantigen reactive B-cells may be activated within the CNS.

Based on the assumption that MS is an (auto)immune mediated disease, most therapeutic approaches are focused on intervention of the aberrant immune response. One of the possibilities to modulate the immune response is the blockade of co-stimulatory signals. Recent research has revealed that a long term immune suppression can be accomplished by the blockade of the gp39 T-cell activation marker and its CD40 ligand present on B-cells. In *chapter 3* we have shown that, the blockade of the gp39/CD40 interaction in EAE mice, by administration of anti-gp39 Abs, resulted in a significant reduction of clinical characteristics of the disease, even when the anti-gp39 monoclonal Abs were administered during disease progression. These results indicate that blockade of gp39/CD40 interactions may be usefull in the treatment of MS as well.

An other approach of immune modulation is suppression of the immune response by induction of tolerance. Tolerance can be induced by oral administration of putative auto-antigens. Live non-pathogenic microorganisms, transformed to produce auto-antigens, may be useful for oral delivery of auto-antigens as they have many advantages over conventional feeding of auto-antigens (*chapter 4.1*). As we shown in a parallel line of research, the presentation of antigens by the

microorganisms to the immune system may also result in the induction of a cellular and/or humoral immune response to the antigens delivered. To generate a safe, non-pathogenic, live vector as an oral antigen delivery system, we have developed a plasmid transformation system for *Lactobacillus* species which are natural inhabitants of the gastrointestinal tract and generally regarded as safe (*chapter 4.2*). In addition we have examined *Lactobacillus* for their ability to deliver antigens via the oral route to the immune system to generate a humoral immune response to a *Lactobacillus* associated model antigen, trinitrophenyl. The results of this study indicate that *Lactobacillus* are potential antigen carriers to generate immunological memory in mucosal as well as in systemical immune responses.

In *chapter 4.3* we describes the routing of *Lactobacillus* associated antigens and the *in situ* localization of antigen specific AFCs in lymphoid organs, after both oral administration and intraperitoneal injection of a *Lactobacillus* trinitrophenyl conjugates. We show that at least a part of the TNP-*Lactobacillus*, is transported directly to the mesenteric lymph nodes (MLN), as could be concluded from the presence of antigen-complexes in MLN tissue sections.

Microbial products with adjuvanticity have been found to play a role in induction of tolerance but can also be used to enhance immune responses in a a-specific manner. Similarly, the adjuvant properties of microorganisms, which are applied as antigen delivery vehicles, may influence the activation or tolerization of the immune system. In *chapter 4.4* we have studied the differential effect of oral administration of antigens, making use of two *Lactobacillus* strains, which differ in their adjuvant properties. In order to induce immunological tolerance *Lactobacillus plantarum 80*, a strain with relatively low adjuvant properties, was administered orally to mice. *Lactobacillus plantarum 80* is transformed for over-production of *E. coli lacZ*, i.e. β-galactosidase. The capacity to mount an immune response was determined after a β-galactosidase challenge injection. The influence of adjuvant properties of microorganisms on the induction of oral tolerance was tested with an autoimmune disease animal model. The effects on the immune system of oral administration of antigen and *Lactobacillus casei*, a strain with high adjuvant properties was studied in EAE mice. The effects of the simultaneous oral delivery of an encephalitogenic epitope and *Lactobacillus casei* prior to disease induction on the course of disease were monitored by evaluation of clinical characteristics. In this study we have shown that oral administration of genetically modified lactobacilli, which secrete antigen, leads to the induction of tolerance. Furthermore, recipient mice become tolerant as well after adoptive transfer of donor spleen cells of the tolerized mice.

The experiments decribed in this thesis show that both blockade of gp39/CD40 interactions and the induction of oral tolerance by application of microorganisms seems rather effective to suppress immune responses in animal model systems. Therefore both methods may have a significant potential for future treatment of autoimmune phenomena in MS patients as well.

SAMENVATTING

Multiple sclerose (MS) is een chronische ziekte van het centrale zenuwstelsel (SZS). Kenmerkend voor de ziekte zijn de ontstekingshaarden en de afbraak van de myelineschede rond de zenuwbanen in het SZS. In de hersenen van MS-patiënten zijn meerdere (multiple) gebieden waarin de myeline is afgebroken als littekens (sclerose) zichtbaar. Ondanks intensief wetenschappelijk onderzoek is de etiologie en pathologie van MS nog steeds grotendeels onbekend. De enorme variatie en complexiteit in klinische verschijnselen maakt het zelfs erg moeilijk om MS te diagnostiseren. Aangezien vele afwijkingen met betrekking tot het immuunsysteem zijn gerelateerd aan de ziekte, wordt in het algemeen aangenomen dat MS het resultaat is van een (auto)immuniteitsreactie.

Het voorkomen van oligoclonale antilichamen in de cerebrospinale vloeistof is een van de (immunologische) kenmerken van MS. Het is echter niet bekend tegen welke antigenen deze antilichamen zijn gericht en op welke wijze deze antilichamen een rol spelen in de pathologie van MS. Om de betrokkenheid van antilichamen in MS te bestuderen is een studie gestart naar antilichaamvormende cellen in de weefsels van het SZS van MS patiënten.

Antilichaamvormende cellen kunnen worden gedetecteerd door gebruik te maken van immuun-histochemische kleuringstechnieken. Voor de toepassing van deze techniek is het noodzakelijk om antigeen-enzym conjugaten te construeren. Tijdens de constructie van dergelijke conjugaten is het essentieel dat zowel het antigeen als het enzym in oplossing blijven. In deze studie is een nieuwe koppelingsmethode ontwikkeld, die het mogelijk maakt om zelfs slecht oplosbare peptide-antigenen te conjugeren.

Er wordt verondersteld dat het immuunsysteem gericht is tegen de structurele eiwitten van de myelineschede in het SZS van zowel MS patiënten als dieren met experimentele allergische encephalomyelitis (EAE), het MS proefdiermodel. Omdat tot nu toe alleen cellen werden gedetecteerd in de cerebrospinale vloeistof of in het bloed en niet in de weefsels van het SZS is het nog steeds onduidelijk of deze structurele eiwitten inderdaad een rol spelen in MS. Het gebruik van enzym conjugaten van myeline componenten maakt het mogelijk dergelijke cellen ook *in situ* te detecteren.

In deze studie is een conjugaat van het enzym peroxidase en een van de hoofdbestanddelen van de myelineschede, nl. het myeline basisch eiwit (MBP), geconstrueerd. Om de veronderstelde rol van antilichaamvormende cellen gericht tegen het MBP eiwit in de pathogenese van MS te bestuderen is het MBP-enzyme conjugaat gebruikt voor de detectie en lokalisatie van anti-MBP antilichaamvormende cellen in een panel van SZS weefsels van MS patiënten, patiënten met andere neurologische ziekten en gezonde personen. De anti-MBP antilichaamvormende cellen zijn alleen waargenomen in het SZS van MS patiënten en niet in de weefsels van het SZS van beide controle groepen.

Voor een effectieve activering van de antilichaamvormende cellen zijn T-cellen noodzakelijk. Binding van het T-cel membraaneiwit gp39 met het CD40 membraaneiwit van de B-cel is essentieel voor de uiteindelijke activering van de B-cel. In deze studie is aangetoond dat gp39 positieve T-cellen aanwezig zijn in beschadigd hersenweefsel van MS patiënten. Dit resultaat is een indicatie dat anti-MBP antilichaamvormende cellen lokaal, m.a.w. in het SZS, geactiveerd kunnen worden.

Omdat verondersteld wordt dat MS een (auto)immuniteitsziekte is, zijn de meeste therapeutische behandelingen van MS gericht op de modulatie van het immuunsysteem. Een van de mogelijkheden om de (afwijkende) immuunrespons te moduleren is het blokkeren van de activeringssignalen tussen de cellen van het immuunsysteem onderling. In deze studie is aangetoond dat de blokkade van de gp39/CD40 interacties in EAE muizen leidt tot een afname van de klinische verschijnselen van EAE, zelfs als de blokkade wordt gestart tijdens de ontwikkeling van de ziekte. Deze resultaten suggereren dat de blokkade van gp39/CD40 interacties als methode van immuun-suppressie toepasbaar is in MS.

Een andere methode van modulatie van het immuunsysteem is het opwekken van tolerantie. Tolerantie tegen (auto)antigenen kan worden geïnduceerd door het oraal toedienen van de vermoedelijke (auto)antigenen. Levende niet-ziekte verwekkende microorganismen, zodanig gemodificeerd dat zij (auto)antigenen produceren, kunnen worden gebruikt voor zowel een effectieve produktie als voor presentatie van antigenen aan het immuunsysteem via de orale route. In een parallelle lijn van onderzoek is tevens aangetoond dat de presentatie van antigenen via de orale route door microorganismen aan het immuunsysteem kan resulteren in de inductie van een immuunrespons gericht tegen het antigeen dat door het micro-organisme is gepresenteerd. Stimulatie van het immuunsysteem dan wel het induceren van tolerantie door gebruik te maken van microorganismen is o.a. afhankelijk van de adjuvant activiteit van het microorganisme die het antigeen produceert/presenteert. Het effect van de adjuvant activiteit op tolerantie inductie/immuun stimulatie is bestudeerd door gebruik te maken van twee *Lactobacillus* stammen die beide verschillen in adjuvant activiteit. In deze studie is aangetoond dat de orale toediening van een genetisch gemodificeerde *Lactobacillus* stam, met een relatief lage adjuvant activiteit, leidt tot de inductie van tolerantie. Tevens is aangetoond dat deze tolerantie kan worden overgedragen door transfusie van de miltcellen van de tolerante muizen naar naïeve ontvanger muizen. Daarentegen leidt de orale toediening van een *Lactobacillus* stam, met een hoge adjuvant activiteit, juist tot stimulatie van het immuunsysteem.

De resultaten van deze studie tonen aan dat zowel de blokkade van gp39/CD40 interacties als de inductie van tolerantie met behulp van microorganismen via de orale route effectief zijn in de suppressie van ziekte in proefdieren. Mogelijk zijn dergelijke methoden van immuun-modulatie ook effectief toepasbaar in MS.

ABBREVIATIONS

Ab antibody
ACTH adrenocorticotropic hormone
ADCC antibody dependent cellular cytotoxicity
AFC antibody forming cells
AP alkaline phosphatase
BALT bronchus associated lymphoid tissues
BSA bovine serum albumin
CFA complete Freunds adjuvant
CFU colony forming units
CNS central nervous system
CR cronic relapsing remitting
CSF cerebrospinal fluid
CT computerized tomography
DAS disability scale
DMF dimethylformamide
EAE experimental autoimmune encephalomyelitis
EDC 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide
ELISA enzyme linked immuno sorbent assay
FCA Freunds complete adjuvant
G gelatine
GA glutaraldehyde
GALT gut associated lymphoid tissues
GL β-galactosidase
GRAS generally recognized as safe
HLA human lymphocyte antigen
HF hydrogen fluoride
HRP horseradish peroxidase
KLH keyhole limpet haemocyanin
Mab monoclonal antibody
MAG myelin associated glycoprotein
MBP myelin basic protein
MHC major histocompatibility complex
MLN mesentheric lymph nodes
MOG myelin oligodendrocyte glycoprotein
MRI magnetic resonance imaging
MS multiple sclerosis
OND other neurological diseases
PBS phosphate buffered saline
PLP myelin proteolipid protein
PVC polyvinyl chloride
RR relapsing remitting
SP spleen
T tween-20
TCR T-cell receptor
Tc cytotoxic T-cell
TD thymus dependent
Th T-helper cell
TNBS 2,4,6-trinitrophenyl sulfonic acid
TNP trinitrophenyl

PUBLICATIONS

1 Boersma WJA, Claassen E, Deen C, Gerritse K, Haaijman JJ and Zegers ND. Antibodies to short synthetic peptides for specific recognition of partly denatured protein. Analyt Chim Acta 213:187-197, 1988.

2 Boersma WJA, Deen C, Gerritse K, Zegers ND, Haaijman JJ and Claassen E. Anti-peptide antibodies as subclass specific reagents: Epitope mapping of human IgG2. Prot Biol Fluids 36:161-165, 1989.

3 Claassen E, Gerritse K, Laman JD and Boersma WJA. New Immuno-enzyme-cytochemical stainings for the *in situ* detection of epitope specificity and isotype of antibody forming B-cells in experimental and natural (auto)immune responses in animals and man. J Immunol Meth 150:207-216, 1992.

4 Deen C, Claassen E, Gerritse K, Zegers ND and Boersma WJA. An improved and modified carbodiimide coupling method for synthetic peptides: Enhanced anti-peptide antibody responses. J Immunol Meth 129:119-125, 1990.

5 Gerritse K, Van Gorcom R, Fasbender MJ, Lange J, Schellekens MM, Zegers ND, Claassen E and Boersma WJA. Specific immuno-detection of benzoate-para-hydroxylase with antibodies raised against synthetic peptides. Biochem Biophys Res Commun 167:33-39, 1990.

6 Gerritse K, Posno M, Schellekens MM, Boersma WJA and Claassen E. Oral administration of TNP-*Lactobacillus* conjugates in mice: A model for investigation of mucosal and systemic responses and immunological memory formation. Res Microbiol 141:955-962, 1990.

7 Gerritse K, Posno M, Fasbender MJ, Schellekens MM, Boersma WJA and Claassen E. Mucosal immune responses and systemic immunological memory after oral administration of TNP-*Lactobacillus* conjugates in mice. In: Imhof B, Berrih-Akhin S, Ezine S, Eds., Lymphatic Tissues and *in vivo* Immune Responses, New York, Marcel Dekker Inc. 84:497-504, 1991.

8 Gerritse K, Fasbender MJ, Boersma WJA and Claassen E. Conjugate formation in urea: Coupling of insoluble peptides to alkaline phosphatase for ELISA and *in situ* Detection of antibody forming cells. J Histochem Cytochem 39:987-992, 1991.

9 Gerritse K, De Knijff P, Van Ierssel G, Havekes LM, Frants RR, Schellekens MM, Zegers ND, Claassen E and Boersma WJA. Immunological discrimination between the human apolipoprotein E2(Arg158→Cys) and E3 isoforms. J Lipid Research 33:273-280, 1992.

10 Gerritse K, Slierendregt B, Deen C, Fasbender MJ, Boersma WJA and Claassen E. *In situ* detection of self reactive epitope specific antibody forming cells in the central nervous system of EAE rhesus monkey. EOS J of Immunol Immunopharma 3:63-65, 1993.

11 Gerritse K, Deen C, Fasbender M, Ravid R, Boersma W and Claassen E. The involvement of specific anti myelin basic protein antibody forming cells in multiple sclerosis immunopathology. J of Neuroimmun 49:153-159, 1994.

12 Gerritse K. Peptide applications in autoimmune disease research. In: Immunological recognition of peptides in Medicine and Biology, Zegers ND, Boersma WJA and Claassen E. 20:269-287, 1995.

13 Gerritse K, Noelle RJ, Aruffo A, Ledbetter JA, Laman JD, Boersma WJA and Claassen E. Functional and histological evidence for the involvement of gp39 (CD40 ligand) in multiple sclerosis. Submitted for publication, 1995.

14 Gerritse K, Maassen K, Leer R, Heemskerk D, Boersma WJA and Claassen E. Lactobacillus as a vector for oral delivery of antigens: The role of intrinsic adjuvanticity in enhancement of immune responses or tolerance induction. Submitted for publication, 1995.

15 Laman JD, Gerritse K, Fasbender M, Boersma WJA, Van Rooijen N and Claassen E. Double immunocytochemical staining for *in vivo* detection of epitope specificity and isotype of antibody forming cells against synthetic peptides homologous to human immunodeficiency virus-1. J Histochem Cytochem 38:457-462, 1990.

16 Zegers N, Gerritse K, Deen C, Boersma W and Claassen E. An improved conjugation method for controlled covalent coupling of synthetic peptides to proteins using glutaraldehyde in a dialysis method. J Immunol Meth 130:195-200, 1990.

17 Zegers ND, Claassen E, Gerritse K, Deen C and Boersma WJA. Detection of genetic variants of α_1-antitrypsin with site-specific monoclonal antibodies. Clin Chem 37:1606-1611, 1991.

CURRICULUM VITAE

De auteur van dit proefschrift is geboren op 3 april 1958 te Rotterdam. In 1978 behaalde hij het H.A.V.O. diploma aan de Caland Scholengemeenschap te Rotterdam. In dat zelfde jaar werd een aanvang gemaakt met een opleiding aan de Stichting Opleiding Leraren te Utrecht. In 1982 werd deze opleiding afgesloten met een 2de en 3de graad onderwijsbevoegdheid in respectievelijk Biologie en Scheikunde. In 1982 werd gestart met een studie Biologie aan de Rijksuniversiteit Utrecht. Hoofd- en bijvakken werden uitgevoerd bij de vakgroepen Stofwisseling Fysiologie en Moleculaire Genetica aan de Rijksuniversiteit Utrecht en bij de vakgroep Immunologie aan de Erasmus Universiteit Rotterdam. In 1987 werd het doctoraal examen behaald. Tevens werd een 1ste graad onderwijsaantekening Biologie verkregen. Vanaf augustus 1987 tot september 1993 was hij werkzaam bij het TNO-Medisch Biologisch Laboratorium op de afdeling Immunologie en Medische Microbiologie, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Vanaf september 1993 is hij als wetenschappelijk medewerker verbonden aan de researchgroep Chemische Toxicologie van het TNO-Prins Maurits Laboratorium.

DANKWOORD

Graag wil ik mijn dank betuigen aan diegene die op enige wijze hebben bijgedragen aan het tot stand komen van dit proefschrift. Van de vele mensen uit werk-, vrienden- en familiekring zijn er een aantal die ik in het bijzonder wil noemen.

Beste Wim, ik wil jou bedanken voor jouw nimmer aflatende steun in zowel goede als slechte tijden. Met name jouw enorme inzet en inbreng bij het vele schrijfwerk in de afgelopen jaren is onvergetelijk. Soms brachten jouw vele suggesties, correcties, verwijzingen, opmerkingen en niet te lezen gekrabbel in de marge mij op de rand van wanhoop. Gelukkig is het toch nog goed gekomen.
Beste Eric, bedankt voor jouw niet te stoppen enthousiasme en creativiteit die ervoor hebben gezorgd dat de motivatie voortdurend op het juiste niveau bleef. De eenvoud en snelheid "*Als je dat experiment vandaag nog even doet....*" waarmee jij ogenschijnlijk van elkaar losstaande experimenten aan elkaar weet te smeden is ongekend. Jij hield altijd de grote lijn in het oog.
Dit proefschrift is het resultaat van een gemeenschapelijke inspanning. Carla Deen, Marianne Fasbender, Diana van Heemskerk, Conny van Holten, Jon Laman, Marjan van Meurs, Louis Ribbens, Mark Schellekens en Netty Zeegers wil ik bedanken voor jullie praktische en/of theoretische bijdrage aan diverse experimenten. Zonder jullie was het absoluut nooit gelukt.
Beste collega's, studenten en stagiaire's van de afdeling Immunologie en Medische Microbiologie, jullie creëerden samen de juiste omgeving waarin naast noeste arbeid ook regelmatig plaats was voor een lekker biertje.
Geen onderzoek vaart wel zonder de hulp van doorgewinterde medewerkers van de spoelkeuken, in het weekend overwerkende dierverzorgers, creatieve fotografen, handige instrumentmakers, slimme electrotechnici, inventieve computerfreaks en snelle secretaresses. Daarom mijn dank aan Michel Boermans, Sjaan Doesburg, Marius Klompenhouwer, Floor Kuylman, Martin Neeleman, Herbert Ramlal, Jeanette Schouw, Henk Tanger, Priscilla Vink en Frans Wijnants.
Pa en Ma, de basis ligt bij jullie.
Beste Pim, jouw niet te evenaren cynische opmerkingen "*Dat opstel komt nooit af*" tijdens het vele knip/plak- en correctiewerk staan voor altijd in mijn geheugen gegrift.

Tenslotte, lieve Karin, wil ik jou bedanken. Veel tijd om samen met jou, Sanne, Else en Rob door te brengen is verloren gegaan. Ondanks dat bleef je mij steunen om dit proefschrift af te ronden. Jij hebt me echt alle kans en tijd gegeven.
Aan jou is dit proefschrift opgedragen.