DOWNREGULATION OF SYSTEMIC IMMUNE RESPONSES BY ORAL AND INTRANASAL ANTIGEN ADMINISTRATION

A dissertation submitted to the University of Cambridge for the degree of Doctor of Philosophy

by

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Wolfson College  September 1994
This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration. It is not substantially the same as any that I have submitted for a degree or diploma or any other qualification at any other university.

[Signature]

September 1994
ABSTRACT

Antigens encountered via mucosal surfaces may specifically reduce systemic immune responses. Oral tolerance, the phenomenon of specific immunological unresponsiveness induced by feeding antigen has previously been demonstrated for food proteins such as ovalbumin (OVA) and target antigens in experimental models of autoimmune diseases. The inhalation of antigens has also been revealed as a potentially tolerogenic route. This thesis investigates mucosal routes to induce immunological tolerance in two models. 1.) Oral tolerance to OVA, 2.) The effect of oral and intranasal administration of myelin basic protein (MBP) and MBP-peptides in a mouse model of experimental autoimmune encephalomyelitis (EAE).

In the Balb/c mouse model of oral tolerance to OVA, feeding 20 mg OVA prior to priming with OVA or the major immunogenic, I-A\[^d\]-restricted peptide 323-339 of OVA reduced specific in vitro lymphocyte proliferation in response to OVA or OVA 323-339 by 40-80%. Transfer of spleen cells or serum from OVA-fed donors under conditions previously shown to suppress delayed-type hypersensitivity responses in recipients, or oral administration of OVA 323-339 alone could not substitute for direct feeding of whole OVA to achieve this effect. The inhibition of specific IgG1 and IgG2a antibody levels argued against immune deviation towards T helper type 2 responses as a relevant mechanism in this model.

In the H-2\[^u\] mouse model of EAE, the dominant T cell response is directed to the acetylated N-terminal peptide Ac1-9 or Ac1-11 of MBP. Analogues of this peptide with alanine or tyrosine at position 4 bind to the I-A\[^u\] MHC class II molecule with higher affinity than the original peptide with lysine at this position, with a hierarchy of affinity Ac1-9 [4Y]>>[4A]>[4K]. In contrast to oral tolerance to OVA, there was no evidence for oral tolerance in the H-2\[^u\] mouse model of EAE when either whole porcine MBP, mouse myelin, or Ac1-11[4K] or [4A] were fed. Abrogation of in vitro T cell proliferation and EAE after oral administration of repeated high doses of the 4Y peptide showed, however, that in principal, encephalitogenic T cells could be targeted by peptide feeding.

Unlike oral administration, a single intranasal 100 µg dose of Ac1-9 (or Ac1-11) or the 4A and 4Y analogues inhibited EAE induced with Ac1-9 alone or whole myelin, showing a positive correlation between peptide-I-A\[^u\] affinity and the degree of protection. These data were consistent with a direct epitope-specific mechanism favoured by high avidity T cell receptor-MHC class II-peptide complexes, independently of (CD8\(^+\)) T suppressor cells. Although inhalation of Ac1-9 and its analogues also protected against whole myelin-
induced EAE, co-immunization experiments using Ac1-9 and the I-Eu-restricted encephalitogenic peptide 35-47 of MBP provided no evidence for bystander suppression. Intranasally administered peptides could also downregulate the functions of encephalitogenic T cells after the induction of EAE, albeit in a yet unpredictable manner which appeared to depend on the state of T cell activation.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac (plus peptide sequence)</td>
<td>Acetyl</td>
</tr>
<tr>
<td>Ac (plus number)</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ADCC AM APC f32M BALT BCR C C (plus number) cAMP CD (plus number) CFA CNS cpm CTL or Tc CY D DAG DC DTH EAA EAE EAU ELISA ER FPLC GALT GM-CSF</td>
<td>alveolar macrophage antigen presenting cell β2 microglobulin bronchial associated lymphoid tissue B cell receptor constant (parts of proteins or genes) complement factor cyclic adenosine monophosphate cluster determinant complete Freund's adjuvant central nervous system counts per minute cytotoxic T cell cyclophosphamide diversity (region of gene) diacylglycerol dendritic cells delayed type hypersensitivity experimental autoimmune arthritis experimental autoimmune encephalomyelitis experimental autoimmune uveoretinitis enzyme-linked immunosorbent assay endoplasmic reticulum fast protein/performance liquid chromatography gut-associated lymphoid tissue granular macrophage colony stimulating factor hen egg lysozyme human gamma globulin human leukocyte antigen high pressure/performance liquid chromatography intranasal(ly) intraperitoneal(ly) intravenous(ly) incomplete Freund's adjuvant interferon immunoglobulin interleukin inositol (tri-, etc)phosphate</td>
</tr>
</tbody>
</table>
Joining (region of gene)
kilo Dalton
keyhole limpid hemocyanin
lipopolysaccharide
microfold cell
monoclonal antibody
mucosal associated lymphoid tissue
myelin basic protein
Major histocompatibility complex
membrane-bound immunoglobulin
mesenteric lymph nodes
natural killer cell
degrees Celsius
ovalbumin
phosphate buffered saline
post capillary venule
phosphatidyl inositol (bis-)phosphate
protein kinase
proteolipid protein
Peyer's patch
purified protein derivative (from mycobacterium tuberculosis)
pertussis toxin
subcutaneous(ly)
spinal cord homogenate
severe combined immunodeficiency
standard deviation
sodium dodecyl sulphate
serum factor
sheep red blood cells
cytotoxic T cell
T cell receptor
tolerogenic (serum) factor
transforming growth factor-β
T helper cell
trinitrochlorobenzene
trinitrophenol
variable (parts of proteins or genes)
CHAPTER 1

Introduction

1.1 SOME PRINCIPLES OF IMMUNE RESPONSES - AN OVERVIEW

1.1.1 The Role Of The Immune System: Discriminating Between The Innocuous And The Detrimental

All biological processes have evolved to secure the survival of the species and maintain the integrity of the individual. Organisms are constantly changing and reacting according to inherent patterns and in response to an environment that may be both hospitable and hostile. The cells and molecules of the immune system pay a major contribution to preserve an individual’s integrity in the light of continuous internal changes and external challenges.

The concept of individual integrity was put forward by Eli Metchnikoff from the embryologist’s point of view, emphasizing the role of the phagocyte to “harmonize potentially discordant cellular elements during development” (Tauber, 1994). The protective role of phagocytes against pathogens was subsequently included in a concept of immunity encompassing both cellular and humoral elements. However, as discussed by Tauber, while Metchnikoff’s concept emphasized integration, the terminology adapted in subsequent years stressed the confrontation between the individual and environmental pathogens culminating in “self-non self” discrimination, one of the most common phrases to characterize the immune system.

Self-nonself discrimination accounts for the majority of components that immune responses should either spare or attack. However, many substances defy this strict categorisation. Some self proteins are only temporarily expressed and may appear as rare and foreign as non-self compounds from the immune system’s point of view. Heterotrophic forms of life require the regular uptake of
foreign matter without inflicting inflammatory reactions against dietary components. On the other hand, immune responses against malignant transformations of self are desirable. Therefore, to account for the dynamics of self, the interplay between non-self and self during metabolism and the occasional conversion from harmless self into malignant self, it is perhaps appropriate to describe the competence of the immune system more generally as being able to distinguish between innocuous and detrimental agents.

1.1.2 Innate And Acquired Immunity

The skin and mucosal surfaces serve as physical barriers to block the entry of pathogens into the body. Mechanical and chemical factors such as ciliary movement and bactericidal components of secreted body fluids contribute to this first line of defence. However, pathogens that manage to defy these obstacles and penetrate a host encounter the complex network of co-operating cells and molecules that constitute the immune system. Some “innate” immune effector mechanisms combat infections without the requirement for specific induction. Phagocytes (polymorphonuclear neutrophils and macrophages), eosinophils and natural killer (NK) cells recognize common glycoprotein and glycolipid structures on the surface of bacteria, parasites or virally infected cells (reviewed in Sastry & Ezekowitz, 1993; Yokoyama, 1993). They destroy pathogens by a wide range of toxic (oxygen) metabolites, oxygen-independent bactericidal and bacteriostatic factors and proteolytic enzymes. Most of these factors are stored in granules ready to be released, either intracellularly upon contact with phagocytosed microbes or extracellularly as a defence against larger parasites. NK cells may initiate programmed cell death (apoptosis) of virally infected cells or destroy such cells by membranolysis following the formation of transmembrane pores lined with NK-derived perforin molecules (reviewed in Berke, 1994).

The complement system provides an important humoral defence mechanism. This names a series of serum proteins organized as an enzyme cascade where one reaction is catalyzed by the product of the preceding reaction allowing for a rapid and highly amplified response to an initial trigger stimulus. The complement pathway can be activated directly by some microbes (alternative pathway) or mediated by antibodies (classical pathway). Both routes merge after the formation of the central molecule C3b. Subsequent products of this pathway (C5-C9) associate with membrane-bound C3b and form a transmembrane
channel through the lipid bilayer, frequently leading to cell lysis (reviewed in Tomlinson, 1993).

Effect mechanisms of innate immunity follow the same reaction patterns with every infection. In contrast, for T and B lymphocytes, a first contact with an infectious agent (primary response) induces significant physiological changes which enable these lymphocytes to respond much faster to a subsequent challenge with the same agent (secondary or memory response). This concept of acquired memory requires lymphocytes to specifically recognize characteristic features beyond those common hallmarks of pathogens that initiate innate immune responses. These individual units of specific lymphocyte recognition, the antigens, bind to receptors exposed on the surface of T and B lymphocytes. They are unique to each lymphocyte (or each clone derived from one lymphocyte) with each receptor being specific for a small part of a particular antigen- the antigenic determinant or epitope. If different antigens share very similar epitopes, these may be recognized by the same antigen receptor: this receptor is then cross-reactive with two or more antigens.

Although T and B cells fulfil different functions, their membrane-bound antigen receptors are similarly organized. They are composed of relatively constant parts forming the overall structure of the molecule and highly variable regions that mediate antigen-specific ligation. Each type of receptor is closely associated with several peptide chains that bear more prominent cytoplasmic domains and link the message of ligand-receptor interactions to intracellular biochemical signalling cascades. These receptor-mediated signals are critical for the development of immature and the functional state of mature lymphocytes (reviewed in Borst et al, 1993).

1.1.3 Antigen Recognition By B Cells

The antigen receptor of B lymphocytes consists of membrane bound immunoglobulin (mIg) and noncovalently associated transmembrane chains (Ig-α, Ig-β) that together with the transmembrane part of mIg mediate signal transduction (reviewed in Cambier et al, 1994). Cognate receptor ligation selects a B cell to proliferate, differentiate and to secrete soluble forms of immunoglobulin (sIg, antibodies) of the same epitope specificity.

The basic structure of immunoglobulins is a four-peptide unit composed of two identical heavy chains and two identical light chains held together by interchain disulphide bonds. Each peptide chain is compactly folded into characteristic globular (Ig) domains. The amino-termini of both heavy and light
chains contribute to the formation of the antigen binding site. They display considerable sequence variability whereas the remaining parts of the chains are relatively constant. Within each variable part some residues display even greater variability and define hypervariable regions which determine the epitope specificity of an immunoglobulin molecule.

Binding of an antigen to the combining site is mediated by the formation of multiple non-covalent interactions which are critically dependent on short distance. This requirement is met when the antigen fits tightly into the combining site. The affinity of antigen-antibody binding is a means to express how well an antigen-antibody fit is achieved, that is how specific this interaction is (reviewed in Amzel & Poljak, 1979).

1.1.4 Antigen Recognition By T Cells

The T cell receptor (TCR) is a non-covalently formed heterodimer consisting of one α and one β chain (or γ and δ chains for some T cells). These chains contain a short cytoplasmic tail, a transmembrane segment and towards the extracellular space one Ig-like and one variable domain. The variable domains of both TCR α and β chains together contribute to the antigen combining site. The TCR forms a complex with a number of small proteins of prominent cytoplasmic regions involved in signal transduction. These are chains of the CD3 complex (γ,δ,ε) and homodimers/heterodimers of ζ and η, respectively (reviewed in Janeway, 1993).

In contrast to immunoglobulins that can bind to epitopes as part of the native three-dimensional structure of the intact antigen, the TCR recognizes a bimolecular complex composed of one small antigenic peptide bound to a molecule of the major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC). APC's (dendritic cells, macrophages, B cells) process antigenic peptides intracellularly either from exogenous and membrane bound proteins or from endogenously synthesized and cytosolic proteins. Along this processing pathway antigenic peptide associates with MHC molecules upon which the MHC-peptide complex is transported to and expressed on the cell surface. Since the association with peptide stabilizes the MHC molecule, most surface MHC products bind intracellularly processed peptide (Sadegh-Nasser & Germain, 1991). However, there are also "empty" MHC molecules on the cell surface which allow for binding of naturally occurring extracellular or experimentally added peptides (Germain & Hendrix, 1991). Furthermore, although many peptides form highly stable MHC complexes, others bind to
products of the MHC with lower affinity and may be replaced by free exogenous peptide at the cell surface (Lanzavecchia, Reid, & Watts, 1992; Fairchild et al, 1993).

There are two classes of MHC molecules. Class I MHC proteins are encoded by three separate genetic loci, H-2K, H-2D and H-2L in the mouse and HLA-A, HLA-B, and HLA-C in humans (reviewed in Guillemot et al, 1988). Each of these loci encodes a single polypeptide chain (the heavy chain) which, when inserted into the plasma membrane displays a short carboxy-terminal cytoplasmic tail followed by the transmembrane segment and a large extracellular part which is folded into three globular domains (α1-α3). They are non-covalently associated with a small non-MHC encoded protein, β2 microglobulin whose structure, like the α3 domain, resembles that of a single immunoglobulin domain.

The class II MHC proteins are heterodimers composed of two non-covalently bonded polypeptide chains, α and β, usually expressed from the same gene locus, either H2-A or H2-E (in mice) for which the products are referred to as I-A and I-E, respectively. In humans the equivalent loci are HLA- DP, DQ, and DR (reviewed in Guillemot et al, 1988). The large extracellular part of each α and β chain is folded into two globular domains, with the membrane proximal α2 and β2 domains displaying the characteristic immunoglobulin fold.

In spite of their different subunit composition, the four globular extracellular domains characteristic for both class I and class II MHC protein complexes form homologous three-dimensional structures. Accordingly, both classes of MHC molecules bind peptides in a principally similar fashion. The two most membrane distal domains, α1/α2 for class I and α1/β1 for class II molecules contribute to the peptide binding groove composed of strands of antiparallel β-sheets forming the floor, and antiparallel α-helical regions as the sides. For class I molecules the boundaries at both ends of the groove restrict the length of peptides that can associate with class I to eight or nine amino acids, whereas the more open-ended sites of class II products permit binding of longer peptides, commonly of 13-17 amino acids. The actual specificity of peptide binding, similar to antigen-antibody interactions, is determined by non-covalent bonds mediated by charged groups, hydrogen bonds, Van-der Waals forces and hydrophobic interactions. However, binding of peptide to MHC molecules is far less stringent than antigen recognition by specific B-and T cell receptors. A limited number of highly polymorphic residues within the binding groove determines the nature of peptides that can form thermodynamically favourable and hence stable interactions with one particular MHC allele. Certain peptide motifs specified by one or more key residues determine the physicochemical properties (size, charge, hydrophobicity) that are best suited to interact with
prominent polymorphic residues characteristic for one particular MHC allele (reviewed in Rotzschke & Falk, 1994).

Generally, peptides that bind to class I or class II molecules are derived from different sources of proteins. For class I, these are usually endosomal self-, viral-, or cytosolic proteins. Binding of peptides to MHC class I molecules occurs in the endoplasmic reticulum (ER). The nascent polypeptide chain of endogenous self or viral proteins may be directly inserted into the ER during proteins synthesis. Alternatively, a complex of proteases, the proteasome, generates peptides from proteins in the cytosol (reviewed in Goldberg, 1992) followed by peptide targeting and transport into the ER by transporter molecules (reviewed in Momburg, Neefjes, & Hammerling, 1994). At this early stage of intracellular MHC molecule trafficking, binding of peptides to MHC class II products is prevented by the invariant chain, a polypeptide that closely binds to and thereby stabilizes class II molecules in the ER and Golgi compartments. While post-Golgi vesicles containing MHC class I-peptide complexes are directly transported to the cell surface, vesicles enclosing free class II molecules fuse with acidic endosomal compartments most of which will include free peptides, lysosomal degradation products of previously endocytosed extracellular or membrane-bound proteins. The low pH of this compartment favours proteolytic degradation of the invariant chain from the αβ class II heterodimer and its exchange with endosomal peptides, which become subsequently expressed at the cell surface, available for T cell recognition (reviewed in Germain & Margulies, 1993). Therefore, in contrast to antigen recognition by B cells which is sufficiently characterized by the bimolecular antigen-immunoglobulin complex, cognate T cell-antigen interactions are based on the trimolecular complex of TCR-MHC-peptide depending on two variables: the affinity of peptide for the MHC (class I or II) molecule and the affinity of the TCR for the MHC-peptide complex.

There are, however, exceptions to this rule of TCR recognition of ‘conventional’ antigens. Superantigens, for example endogenous mouse mammary tumour virus and some exogenous bacterial toxins may associate directly with extracellular MHC domains rather than being processed and presented within the peptide binding groove. As a consequence, the mode of antigen recognition by the TCR is also different and largely governed by the variable part of the β chain (Vβ) alone (reviewed in Marrack et al, 1993). Since many T cells specific for a variety of different conventional antigens may nevertheless share the same Vβ, superantigens can mediate polyclonal T cell-antigen interactions, in sharp contrast to generally uniclonal T cell recognition of individual types of MHC-peptide complexes.
1.1.5 Developing And Co-ordinating Immune Responses

1.1.5 i Generation of antigen-receptor diversity

The problem of generating specific recognition sites for a virtually unlimited number of epitopes from a limited number of antigen receptor genes and their alleles is solved by the unique mechanism of gene rearrangement at early stages of B and T cell development (reviewed in Blackwell & Alt, 1988; Davis, 1988). Multiple clusters of gene segments code for the variable (V) and constant (C) regions of antigen receptors. These genes enclose additional segments encoding the joining (J) region and, in the case of the immunoglobulin heavy chains and the TCR β chain also diversity (D) regions. Within the embryonic germ line DNA of B and T cell precursors these gene segments are separated by long intervening nucleotide sequences which become enzymatically excised when a V gene is translocated to lie next to a J (or previously combined JD) segment, generating VJ-intron-C or VJD-intron-C of B or T cell DNA. This VJ or VJD segment will determine the epitope specificity of the successfully rearranged and transcribed antigen-receptor gene product. The intron between VJ(D) and C is excised prior to transcription of the functional antigen-receptor molecule.

Theoretically, the number of different antigen-receptors that can be generated by this mechanism is given by the product of the number of V, J, D, and C segments. Although a diploid set of these genes is available, after the successful rearrangement of one set of genes, the corresponding allelic genomic sections are inactivated (allelic exclusion), thereby ensuring unique antigen specificity for each individual lymphocyte. However, at the nucleotide level, additional diversity can be achieved by somatic mutation, some variation in the exact site of the V-J-D join ('hot spots'), and somatic hypermutation as a peculiar phenomenon during the maturation of a B cell response. Further diversity of the TCR repertoire arises from the insertion of nucleotides at the N region of the D and J segments (reviewed in Blackwell & Alt, 1988; Davies, 1988). At the protein level, the antigen-receptor monomers may form different combinations of the complete multimeric antigen-receptor.

1.1.5 ii The role of APC’s and coreceptors

While the antigen receptors confer specificity a multitude of other surface molecules expressed on lymphocytes and APC’s are crucially important for the
development and regulation of lymphocytes. Receptor-coreceptor interactions between these cells not only increase the degree of physical intercellular adhesion; their cytoplasmic domains are often integral parts of signalling pathways, thereby regulating intracellular biochemical events.

The variable capacity of different types of APC’s to induce T cell activation is largely based on their ability to provide costimulatory signals mediated by such coreceptors. Among those, the receptor/counterreceptor pair B7/CD28 (or B7/CTLA-4) provides a critical costimulatory signal for the activation of naive (antigen-inexperienced) T cells (reviewed in Schwartz, 1992). Furthermore, the functions of MHC molecules extend beyond mere peptide presentation. Their cytoplasmic tails are involved in intracellular signalling events that induce and upregulate the expression of costimulatory molecules (reviewed in Wade et al, 1993). Thanks to their high levels of surface MHC class II and their ability to constitutively express B7, dendritic cells (DC) are the primary APC for the activation of naive T cells. Other APC’s such as macrophages can be induced by IFNγ to upregulate levels of class II MHC and to express B7. Primed B cells also function as APC and can provide costimulation upon activation. Their antigen receptor enables them to take up antigen specifically and therefore more efficiently than other types of APC’s. In turn, coreceptor signals and T cell-derived lymphokines (T cell “help”) govern further B cell functions. Apart from B7/CD28, a whole range of other surface molecules such as LFA-1/ICAM-1, CD40/CD40L, CD2/CD58 or CD59 and others contribute to intercellular adhesion and intracellular signalling (reviewed in Clark & Ledbetter, 1994).

1.1.5 iii Intracellular signalling events

The coreceptors that become most closely associated with the TCR/CD3-MHC-peptide complex are CD4 and CD8 which are alternatively expressed on the surface of mature T lymphocytes. CD8 associates with MHC class I molecules whereas CD4 binds to MHC class II molecules. These interactions therefore specify two classes of T cells with respect to the source of antigenic peptides they encounter: CD4-positive T cells generally are directed to class II MHC molecules in combination with peptides captured in acidic endosomal compartments (although there may be exceptions), whereas T lymphocytes expressing the CD8 receptor are destined to encounter peptides largely derived from endogenous- or cytosolic proteins.

T cell activation initiates at least three inter-linked second messenger pathways that are tightly controlled by Ca2+- dependent reactions and phosphorylation / dephosphorylation of certain key regulatory enzymes. These
control mechanisms constitute complex positive and negative feedback loops ensuring appropriate and prompt reactions by either rapid signal amplification or downregulation. Tyrosine kinases of the src and 70kd family which are associated with the cytoplasmic domains of both CD4 and CD8, and chains of the TCR/CD3 complex respectively, act coordinately to recruit by tyrosine phosphorylation a complex of molecules that may specifically interact with the phosphorylated form of the cytoplasmic receptor domains and couple the TCR to downstream intracellular events. Although the precise sequence of subsequent events is yet poorly defined they involve further kinases and phosphatases which are themselves regulated by phosphorylation and/or Ca$^{2+}$ (reviewed in Janeway, 1993). Recent evidence suggests that the src tyrosine kinases which initiate this cascade are themselves subject to regulation by CD45, an abundant leukocyte surface protein with tyrosine phosphatase activity (reviewed in Trowbridge & Thomas, 1994).

Phospholipase C$_{Y1}$ is another key enzyme triggering early events of T cell activation. It may bind to the tyrosine-phosphorylated cytoplasmic domains of the TCR upon which it is itself phosphorylated, thereby increasing its affinity for its substrate phosphatidyl inositol-bisphosphate (PIP$_2$). Breakdown of PIP$_2$ generates diacylglycerol (DAG) and inositol-triphosphate (IP$_3$), two central mediators of subsequent intracellular signalling. IP$_3$ releases Ca$^{2+}$ from intracellular stores. Together, DAG and Ca$^{2+}$ activate protein kinase C (PKC) which in turn mediates the activation and translocation of nuclear factors. The central role of Ca$^{2+}$ in the regulation of both PKC and src/70kd-initiated signalling pathways demands a tight control over intracellular calcium levels. This is largely achieved by the metabolism of IP$_3$. Removal of IP$_3$ not only prevents further release of Ca$^{2+}$ from intracellular stores but, furthermore, other inositol-phosphate metabolites such as IP$_4$ are actively engaged in replenishing these stores (reviewed in Berridge, 1993).

In addition to the downregulation of positive signals, TCR ligation also initiates negative regulation mediated by the serine-threonine protein kinase cAK1, an enzyme that is functionally dependent on cyclic adenosine monophosphate (cAMP). cAK1 co-localizes with the TCR/CD3 complex upon TCR ligation and may establish an inhibitory signalling pathway that interferes with protein phosphorylation by TCR/CD3-associated tyrosine kinases (Skalhegg et al, 1994).

Among the first proteins expressed as a result T cell signalling are the interleukin-2 receptor and interleukin-2 (IL-2). IL-2 is a crucial autocrine growth factor for the proliferation and differentiation of all T lymphocytes. However, engagement of the TCR/CD4 or CD8 complex with peptide-MHC alone is not
sufficient to initiate T cell activation. As mentioned above, synergy with costimulatory molecules is required for IL-2 production in a primary immune response.

The events following specific B cell receptor (BCR) ligation appear to include very similar mechanisms outlined above for TCR ligation-mediated intracellular signalling cascades (reviewed in Borst, 1993; Cambier et al, 1994). Homologous to signal transduction via TCR-CD3 and CD4/CD8, the Igα and Igβ chains together with coreceptors including CD22 and CD19/CD21 mediate proximal activation of protein tyrosine kinases such as src and lyn, GTP-binding proteins (p21ras), more distal serine/threonine kinases all which by various diverging biochemical cascades serve to convert the message of BCR ligation into gene transcription.

1.1.5 iv Regulation of effector functions

The variable nature of pathogenic insults requires selective acquisition of those effector functions that exactly meet the needs of the infected organism to successfully combat the intruder. There is no general rule to attribute a particular immune response to one particular pathogen. Rather, the route of entry, the tissue or cell type that become infected and whether or not the immune system has encountered the pathogen before, together with the actual antigenic “make up” of the infectious agent, determine the nature of the immune response. However, for immune responses against any given infection, it is often appropriate to be dominated by either antibody- or cell mediated mechanisms. At the effector stage, many features of innate and acquired immunity converge.

The effector functions of antibody molecules are mediated by the constant Fc (“fraction crystalizable”) domains of the heavy chain. These enable antigen-antibody complexes to activate complement by the “classical” pathway thereby attacking infectious agents that fail to activate complement directly. The Fc portion also specifically binds to Fc receptors on the surface of effector cells thus targeting and enhancing phagocytosis of (antibody-complexed) bacteria. Furthermore, antibodies that bind to pathogen-derived antigens expressed on the surface of infected cells may trigger cytotoxic mechanisms by which effector cells are targeted to the antibody-coated cell via their Fc receptors in order to kill the target directly (antibody dependent cell mediated cytotoxicity, ADCC).

T cell mediated immune responses are involved when infectious particles gain access to MHC class I and/or class II processing pathways. Cytotoxic cells (Tc or CTL) specific for these MHC-peptide complexes may destroy the infected target cell directly by two mechanisms that are also characteristic of NK cells: by
necrosis (membranolysis) probably mediated by perforin lined transmembrane pores leading to osmotic lysis or by apoptosis (reviewed in Berke, 1994). Tc cells are usually CD8-positive and MHC class I-restricted. However, CD4-positive T cells can also acquire a cytotoxic phenotype. Another mechanism mediated by CD4+ T cells is delayed type hypersensitivity (DTH). Here, the interaction of APCs (presumably mainly or exclusively macrophages) presenting antigen to primed CD4+ T cells culminates in an inflammatory response: Cytokines are released that further attract and activate macrophages to release lysosomal enzymes and reactive oxygen metabolites.

Most of these effector functions require primed lymphocytes, those that have already encountered antigen before (memory cells). A first confrontation with (T-dependent) antigens induces CD4+ T helper (TH) cells to control the development of subsequent immune responses. T helper functions are mainly governed by cytokines. This has been particularly well described for the murine immune system. Here, two populations of TH cells, TH1 and TH2 control the nature of the immune response by secreting characteristic and mutually antagonistic sets of cytokines. TH1 cells produce IL-2 and IFNγ whereas IL-4, IL-5, IL-6 and IL-10 are characteristic of TH2 cells. Both types of TH cells can produce IL-3, tumour necrosis factor α (TNFα) and granular macrophage colony stimulating factor (GM-CSF). This distinction based on lymphokines corresponds to distinct functional phenotypes (reviewed in Mosmann & Coffman, 1989). TH1 cells appear to mediate DTH reactions and activation of CTL whereas TH2 type lymphokines favour antibody-mediated effector mechanisms.

With respect to T helper functions for B cells these lymphokines control the mechanism by which the VDJ segment of the immunoglobulin heavy chain is linked to one of several constant region genes for the expression of the soluble immunoglobulin molecule (class switching). While the rearranged VDJ segment define antigen specificity, the constant regions mediate different effector functions and define the immunoglobulin isotype. Different isotypes vary in their ability for ADCC, complement fixation, and they may selectively bind to different types of Fc receptors (reviewed in Greenwood & Clark, 1993; Morrison, Canfield & Tao, 1993). The process of class switching thus allows the same antigen specificity to be combined with various different effector functions to adapt to changing host requirements as an immune response evolves. For instance, in the mouse, IL-4 (from TH2 cells) selectively induces switching to the e and γ1 loci whereas TH1 type IFNγ inhibits IL-4 induced switching and causes switching to γ2a. IgG1 and IgE levels become elevated during nematode infections. They bind well to Fc receptors on monocytes and mast cells and are
therefore efficient mediators of ADCC (reviewed in Snapper & Mond, 1993). Some bacterial antigens such as lipopolysaccharide (LPS) may crosslink mIg non-specifically and activate B cells independently of T cell help. However, T-independent antigens usually only trigger IgM production and lack the ability to induce class switching or affinity maturation of an antibody response.

TH1 and TH2 cells do not constitute fixed pre-formed sets of lymphocytes, but appear to emerge from a common, relatively unspecialized TH0 phenotype. The differentiation into either TH1 or TH2 cells appears to depend on the cytokine environment in which a T helper cell first encounters antigen. In the presence of IL-4 T cells also produce IL-4 upon restimulation whereas the development into TH1 effectors is inhibited by IL-4. Conversely, in the absence of IL-4, an IFNγ-producing T cell phenotype develops (reviewed in Seder & Paul, 1994). A certain cytokine environment is often created by the infectious agent itself and effecter cells of innate immunity. IFNγ-secreting NK cells and IL-12 produced by Listeria-infected macrophages synergized to induce a TH1-type response against bacterial infection (Hsieh et al, 1993). Experimental Leishmania major infection in mice induced distinct T cell functions corresponding to either a TH1 or a TH2 phenotype which could be correlated with either recovery and survival or a lethal infection, respectively (Scott et al, 1988; Heinzel et al, 1989). In contrast, immunity against helminth worms such as schistosomes was dominated by IgE-mediated ADCC. Here, one egg component, a glycoprotein appeared to induce B cells to proliferate and to secrete IL-10 (Velupillai & Harn, 1994).

1.1.6 The Need For Immunological Tolerance

Most effecter functions culminate in inflammatory responses which, besides destroying the pathogen, often also cause some local tissue injury. Since effecter mechanisms are tightly controlled and rapidly downregulated after the successful removal of the pathogenic insult, this is usually an acceptable price to pay. However, immune responses can only be constructive if the destructive forces are exclusively directed against hazardous agents. Since the vast majority of MHC molecules contain peptides derived from the organism’s own proteins (self-peptides) this seems not a trivial problem. In order to meet the requirement for selective immunity, T lymphocytes need to interpret TCR-MHC-peptide ligation in different ways so as to direct effecter functions against detrimental while tolerating innocuous antigens.
Immunological tolerance can be induced in both B and T lymphocytes. However, for all T-dependent antigens (to which all antigens studied in this thesis belong), tolerance in the T cell compartment will also abrogate B cell-mediated responses to these antigens. The next section discusses various strategies by which T cell tolerance is achieved.

1.2 IMMUNOLOGICAL TOLERANCE

1.2.1 Modes Of Tolerance Induction

One way to avoid destructive immune reactions against innocuous agents is the elimination of T lymphocytes bearing specific receptors for such antigens. Alternatively, T cells may be rendered unable to convert the message of cognate TCR-peptide- MHC ligation into activating biochemical signals; they are said to be anergic. Among mature lymphocytes, regulatory networks in which some (suppressor) T lymphocytes modulate the functions of other T cells also play a significant role.

All three mechanisms, deletion, anergy and suppression have been demonstrated for the control of mature lymphocytes in the periphery. The education of immature lymphocytes follows a rigorous regime mainly leading to deletion of T lymphocyte precursors that fail to withstand certain selective pressures. The central organ for the education of immature T lymphocytes is the thymus. To account for the different developmental stages of T lymphocytes at the time of tolerance induction and the central role of the thymus, rules that govern T cell tolerance are conveniently discussed as central (thymic) and peripheral tolerance.

1.2.2 Central Tolerance

Early T lymphocyte precursors that enter the thymus express neither TCR nor CD4/CD8. As they traverse the thymus they either die or differentiate under various selective pressures (reviewed in Robey & Fowlkes, 1994). Thymocytes develop into early double positive cells with low levels of TCR, at which stage they are positively selected for their ability to bind to self-MHC-peptide complexes (MHC restriction), while all other thymocytes are programmed to die. Furthermore, T cells commit themselves to one lineage defined by the CD4 and
CD8 coreceptors. Consequently, T cells that bind to peptide-MHC class I complexes only express CD8 and, conversely, specific interactions of the TCR with MHC class II and peptide are associated with surface expression of CD4. During the subsequent stage of negative selection a high proportion of lymphocytes is deleted upon cognate MHC-peptide interactions.

Negative selection in the thymus eliminates a high proportion of self-reactive T lymphocytes and therefore pays a major contribution to self tolerance. However, most self and nonself proteins are too similar in sequence to allow for a self-nonself discrimination based on qualitative differences between proteins by either APC’s or T cells. Rather, the nature of peptides that can be processed and presented is determined by constraints imposed by the MHC peptide binding groove, protease specificities and (for class I molecules) transporter molecules (Driscoll et al, 1993; Powis et al, 1992). Therefore, self-reactive T cells are not negatively selected for the recognition of “self” as a qualitative entity per se but because they do not normally encounter “nonself” in the neonatal thymus. Or, to phrase this differently: because the majority of innocuous antigens are indeed self proteins, the thymus as a specialized organ of densely packed complexes of self MHC molecules and representative self peptides guarantees the deletion of those self-reactive immature T cells which, as mature T cells in the periphery could be expected to induce fatal autoimmune responses.

The apparent paradox as to how recognition of self MHC-self peptide complexes direct T cell survival during positive selection and at later stages induce programmed cell death (apoptosis) has been an enigma for a long time. Clearly, two requirements for thymocyte selection have to be met. 1) The neonatal thymus expresses only self peptides. Obviously, however, the deletion of all self-reactive T cells would thwart the purpose of the immune system. Therefore, negative selection should only delete thymocytes with specificity for those self-peptides that could potentially induce detrimental T cell-mediated reactions in the periphery. 2) Although T cells can only be positively selected by self peptides, they need to recognize pathogenic antigens not previously expressed in the thymus. This requires positively selected T lymphocytes with specificity for “self” to be sufficiently cross-reactive with “nonself”. Or- more pointedly- in order to combat pathogens while sparing innocuous self, T cells have to respond to certain peripheral antigens with considerably greater efficiency than to those antigens that they were originally selected for.

To explain the paradox that self MHC-self peptide complexes induce both positive and negative selection, several mechanisms have been proposed. Different thymic tissues and types of APC were suspected to selectively mediate either positive or negative selection. However, numerous studies demonstrated
that thymocyte selection was not crucially dependent on intrinsic properties of different types of APC’s. When antigen presentation by endogenous thymic tissue was abrogated, different lines of fibroblasts could sufficiently reconstitute positive selection, both with respect to MHC restriction and peptide-specific selection (reviewed in Pawlowski & Staerz, 1994). It also became clear that APC from many different sources including professional peripheral APC’s could induce deletion of maturing T lymphocytes (Matzinger & Guerder, 1989). Furthermore, thymus grafting experiments with chimeric recipients suggested that, although different thymic tissues might display distinct avidity interactions between maturing T lymphocytes and thymic APC’s, Cognate, yet low avidity ligation would rescue thymocytes from apoptosis and enable the progress of development. Subsequently, T cells capable of forming high avidity interactions would be deleted (Pircher et al, 1991). The overall avidity of T cell-APC interactions is a function of both ligand-receptor affinities and concentrations. If the avidity model were valid, then manipulating any of these variables should influence the outcome of thymic selection.

Several recent reports are consistent with the avidity model of thymocyte maturation. In these studies the differentiation of thymocytes bearing transgenic TCR specific for a model peptide was monitored in fetal thymic organ cultures. The sensitivity of thymocyte responses to exogenous peptides was sufficiently increased by using mutants with defects in MHC complex formation with endogenous peptides, either due to β2M or peptide transporter (TAP1) deficiencies. With these methods it was possible to demonstrate that different concentrations of the same peptide (10^-6 M versus 10^-12 M) could induce either negative or positive selection with β2M^- APC’s (Sebzda et al, 1994). When peptide was added to TAP1^+ and TAP1^- cultures, negative selection required higher peptide concentrations with TAP^- than with TAP^+ APC’s (Ashton-Rickardt et al, 1994). Essentially the same message arose from experiments with another peptide in β2M^- versus β2M^+ cultures (Hogquist et al, 1994). These findings suggested that the summations of TCR-MHC-peptide interactions determine the outcome of T cell differentiation in agreement with the avidity...
model. Another report proposed that the expression of coreceptors that contribute to the overall T cell-APC avidity may also determine the outcome of T cell selection. An increase in the surface expression of CD8 induced negative selection while moderate to low levels of surface CD8 allowed for positive selection of T cells specific for the same peptide (Robey et al, 1992).

1.2.3 Properties Of Peripheral T Lymphocytes

The evidence in favour of the avidity model for thymic selection allows one to predict the properties of T cells that escape thymic deletion and are released into the periphery. Generally, they can be expected to form low avidity TCR-self MHC-self peptide interactions, either due to low intrinsic ligand-receptor affinities or because the concentrations of interacting molecules are low, in particular the concentration of peptides available for presentation.

1.2.3 i Dominant, subdominant, and cryptic epitopes

The rules that govern antigen handling by APC’s restrict the number of peptides that can be processed and presented. In consequence, these peptides dominate T cell responses to any given antigen. Other peptides may also be presented, albeit less efficiently. These constitute minor or subdominant antigenic determinants. In more extreme cases, antigenic peptides are presented with such low efficiency that T cell responses to these cryptic epitopes are virtually undetectable upon challenge with the intact antigen except at very high concentrations. Experimentally, a hierarchy of dominance among several epitopes can be assessed by priming and restimulating T lymphocytes with the whole protein or synthetic peptides representing different regions of the complete sequence. If peptide-primed T cells can be restimulated with the intact antigen, then this peptide necessarily contains at least one of the naturally processed epitopes. Cryptic peptides may elicit vigorous T cell responses to themselves but cannot be recalled after priming or restimulation with the intact protein (reviewed in Ametani et al, 1989). The ability to elicit strong immune responses to cryptic and minor epitopes with these peptides alone also suggests that a hierarchy of dominance among epitopes is determined by the APC rather than the lack of specific TCRs. The avidity model of thymic T cell differentiation therefore predicts that clonal deletion eliminates self-reactive T cells specific for dominant epitopes and permits the maturation of T cells specific for minor or cryptic self epitopes. Before the avidity model had gained further support, this
idea was formulated as a “threshold escape hypothesis” by which autoreactive T cells specific for dominant self epitopes would be deleted while allowing the escape of T cells specific for minor or cryptic epitopes (Gammon, Sercarz & Benichou, 1991).

1.2.3 ii Low affinity peptides

Epitope subdominance as defined above is not a prerequisite for the escape from central tolerance induction. In a mouse model of an experimental autoimmune demyelinating disease (which is also the subject of this thesis) the dominant peripheral T cell response is directed against a peptide of mouse myelin basic protein that forms extremely unstable complexes with its specific MHC class II restriction element. The authors propose that the very low affinity interactions between peptide and MHC molecules prevent thymic deletion (Fairchild et al, 1993). Here, the “threshold escape” is defined by the low affinity of ligand- MHC interactions rather than by the low concentrations of cryptic or subdominant peptide ligands. However, both cases amount to the same net result, namely low concentrations of TCR-MHC-peptide complexes at any given time thereby reducing the overall avidity below a critical threshold for thymic deletion.

1.2.4 Peripheral Tolerance

1.2.4 i Positive correlation between epitope dominance and peripheral tolerance

The existence of peripheral peptides which were not available for thymic presentation requires that immunological tolerance can also be induced in mature thymic emigrants. Peripheral tolerance is established and maintained by various (not mutually exclusive) mechanisms such as deletion, anergy, and suppression depending on the model and experimental conditions. For some phenomena of peripheral tolerance that are sufficiently explained on the basis of direct T cell-APC interactions (i.e. without the need for suppressor cells), there appears to be a parallel with central tolerance. Adult mice rendered tolerant to hen egg lysozyme (HEL) failed to respond to whole HEL or immunodominant HEL peptides, while T cell activation with subdominant epitopes was still possible. These findings suggested that those epitopes that dominated T cell activation were also dominant in tolerance induction. Epitopes that failed to activate T cells efficiently would also be poor tolerogens (Gammon & Sercarz,
1989). A transgenic mouse model of endogenously expressed HEL confirmed these observations that central and peripheral tolerance were more easily achieved with the dominant HEL epitope. However, within the hierarchy of dominance of three HEL peptides (108-116>>1-18>74-96), tolerance to the subdominant epitopes was also observed in mice expressing high serum levels of endogenous HEL. The positive correlation between epitope dominance and tolerogenicity on the one hand and levels of peripheral HEL required to enable the tolerogenicity of subdominant epitopes on the other hand suggested that the actual number of specific peptide-MHC complexes displayed on APC was decisive (Cibotti et al, 1992). It is therefore conceivable that high avidity TCR-MHC-peptide interactions also determine peripheral tolerance in some models.

In these studies T cell tolerance to HEL was assessed by the ability of primed lymphocytes to mount specific proliferative responses in vitro. This method allowed the analysis of the relative significance of individual epitopes in detail. It did not, however, distinguish between anergy and deletion since lower levels of proliferation may reflect an overall reduction in the number of responder cells or the inability of anergic T cells to proliferate in response to an antigenic stimulus. Due to the low numbers of precursor T lymphocytes with specificity to any one conventional antigen in a physiological T cell repertoire (about 1/10^5), it is usually impossible to follow the fate of individual T cell clones to distinguish between deletion and anergy.

1.2.4 ii Deletion and anergy through activation

The limitations set by low T cell precursor frequencies were circumvented in models utilizing transgenic TCR or superantigens. Studies on mouse models with transgenic TCR specific for the male H-Y antigen (Rocha & von Boehmer, 1991) or the superantigen Mlsα (Webb, Morris, & Sprent, 1990) showed that the constant confrontation of self-reactive T cells with high densities of specific antigen in the periphery evoked a characteristic kinetic pattern of rapid in vivo expansion followed by a steep decline. Furthermore, the remaining H-Y- or Mlsα-specific peripheral T cells were unresponsive to subsequent antigenic stimulation in vitro, and they did not proliferate in vivo after secondary transfer. This process was progressive and could be correlated with the downregulation of TCR and CD8 on the T cell surface (Webb, Morris, & Sprent, 1990). The same sequence of events of tolerance through activation was also observed in transgenic models using more conventional antigens such as viral peptides (Mamalaki et al, 1993; Moskophidis et al, 1993). The mechanism of tolerance in
these studies was interpreted as clonal exhaustion of T cells as a result of long-term exposure to antigen.

This sequence of proliferation, deletion and irreversible anergy of remaining T cells is not a universal mechanism of peripheral tolerance. Deletion may be observed without prior proliferation (McCormack et al, 1993; Waanders et al, 1993). Furthermore, when the peripheral concentrations of self-antigen were lower such as in heterozygous mice expressing only half the amounts of a specific Mls superantigen, clonal deletion of Mls-reactive T cell clones was incomplete, and clonal anergy of the remaining responder T cells was reversible in hosts that lacked the particular superantigen altogether (Ramsdell & Fowkes, 1992). The ability to reverse anergy in the absence of antigen was confirmed for T cell clones specific for the male antigen H-Y upon transfer into female recipients (Rocha, Tanchot & von Boehmer, 1993). When the level of peripheral antigen expression was under the control of an inducible promoter, T cell tolerance as assessed by the downregulation of TCR surface expression was partial at low and complete at high antigen concentrations (Ferber et al, 1994). These studies highlighted the importance of the antigen dose in determining the mode of peripheral tolerance induction.

1.2.4 iii The role of APC's in peripheral tolerance

Numerous reports highlight the significance of different APC's or different physiological states of the same APC for peripheral tolerance. Furthermore, the nature of the antigen and the route of antigen administration may determine the outcome of immune responses. Some phenomena of peripheral nonresponsiveness can be explained by antigen presentation in the absence of costimulation or by selective induction of TH2 cells that subsequently inhibit the induction of a TH1 type response. All these factors may be closely inter-linked: the route of antigen administration may determine the type of APC that will be confronted with the antigen, and APC's may differ in their ability to provide costimulation or preferentially induce either TH1 or TH2 type responses.

Anergy in the absence of costimulatory signals

An early explanation for clonal anergy has been the presentation of antigen in the absence of costimulatory signals. Antigen presentation by fixed (physiologically inactivated) APC's in vitro resulted in functional inactivation of T cell clones as shown for proliferation and IL-2 production. However, the addition of normal allogeneic APC's, themselves incapable of presenting antigen, abolished this effect. Experiments in which semipermeable membranes
separated fixed and unfixed APC’s demonstrated that soluble mediators were not responsible for this phenomenon (reviewed in Mueller, Jenkins, & Schwartz, 1989). Thus, it appeared that the quality and quantity of physical contacts between APC’s and T cells beyond those mediated by the MHC restriction element constituted critical parameters for the modulation of T cell activity. The lack of costimulation seemed not only to prevent the induction of effector mechanisms but to actively induce negative regulation that compromised subsequent T cell functions in the presence of costimulatory signals. Studies on an autoimmune disease model (experimental autoimmune encephalomyelitis, EAE) appeared to confirm the significance of lacking costimulatory signal for tolerance induction in vivo. Here, intravenous administration of encephalitogenic peptide coupled to fixed splenocytes protected animals from disease (Pope, Paterson & Miller, 1992; Tan, Kennedy, & Miller, 1992). Likewise, mice treated with encephalitogenic peptide coupled to soluble MHC class II molecules were also protected against EAE. (Sharma et al, 1991).

While isolated MHC-peptide complexes and the fixation of APC’s are experimental manipulations to deprive T cells of costimulation, APC’s that are devoid of essential coreceptors under physiological conditions might “naturally” inhibit those immune responses that are critically dependent on coreceptor-mediated signals. In search for a candidate APC that induces tolerance in vivo, the B cell became the focus of attention for a series of studies. Much of the T cell-B cell cross-talk depends on the activation state of both types of lymphocytes since several coreceptors are not expressed on resting or naive B and T cells. While dendritic cells can prime naive T cells, B cells can only stimulate T cells during a secondary response (Ronchese & Hausmann, 1993). One study investigated the responses of naive and memory T cells from female donors to resting and activated B cells presenting the male-specific antigen H-Y. Both resting and LPS-activated B cells were tolerogenic for naive T cells whereas memory T cells were stimulated even when the B cells were not previously activated (Fuchs & Matzinger, 1992). When antigen was specifically targeted to B cells by anti-IgD, T cell functions such as in vitro proliferation, IL-2 production and TH-dependant antibody production were diminished, if the tolerizing protocol was applied before or at the time of the antigenic challenge (Eynon & Parker, 1993). The tolerogenic potential of B cells was also explored in a rat model of EAE demonstrating protection from EAE when an encephalitogenic peptide was linked to anti-IgD for B cell targetting (Day et al, 1992). Taken together, these results suggested that the outcome of the T cell-B cell dialogue was critically dependent on the T cell’s history and emphasized the
competence of B cells to induce antigen-specific tolerance in naive T lymphocytes.

**Differential requirements for tolerance induction in TH1/TH2 subset**

A number of reports showed that antagonistic cytokines characteristic of TH1 or TH2 responses could guide T cell functions towards either activation or tolerance. Beyond the failure to provide costimulation under some conditions, the tolerogenic effect of B cells as APC's was also attributed to a preferential induction of TH2 responses accompanied by selective inhibition of TH1 cells. In one report, the authors speculated that the combination of impaired in vitro TH1 responses (proliferation, IL-2, IFNγ, and serum IgG2a) and increased levels of IL-4 after intraperitoneal injection of human gamma globulin(HGG) might have originated from Fc-Fc-receptor mediated antigen targetting to B cells. (De Wit et al, 1992). Other studies examined the requirements for tolerance induction with established TH1 and TH2 clones in vitro. Chemical fixation of APC's only rendered a TH1 clone unable to proliferate in response to an antigenic stimulus while the proliferative response of a TH2 clone was unaffected. However, clones of both TH subsets were unable to provide B cell help for antibody production (Gilbert, Hoang, & Weigle, 1990). When the significance of different APC's for selective T cell activation was addressed, TH2 clones seemed to proliferate preferentially in response to B cells as APC's whereas a TH1 clone was optimally activated by plastic adherent splenocytes, mostly macrophages and dendritic cells (Gajewski et al, 1991). To pursue the question of differential TH1/TH2 regulation further, the lymphokine profiles of TH0, TH1 and TH2 clones were examined in vitro in response to treatment with anti-CD3 as a protocol for anergy induction. This method selectively inhibited TH1 functions such as IL-2 production and cytolytic activity while anergy could not be induced in TH2 clones. Significantly, the TH0 clones which exhibited a mixed cytokine profile (IL-2, IL-4, IL-5) prior to the incubation with anti-CD3 specifically lost the ability to exert TH1 type functions while retaining a TH2 phenotype (Gajewski et al, 1994). Together, these results suggest that Th1 and Th2 type responses follow different rules both for specific activation and tolerance induction.

However, the evidence for differential induction of anergy in TH subsets in vitro remains spurious for the in vivo situation. In one study, inactivated Theiler's virus coupled to fixed splenocytes and injected i.v. selectively inhibited TH1 but not TH2 responses as assessed by DTH, proliferation, lymphokine production, and the secretion of characteristic immunoglobulin isotypes. These findings suggested that lack of costimulation selectively inhibited TH1 responses...
and were interpreted as "split tolerance" (Peterson et al, 1993). However, it may be difficult to rigorously prove in vivo whether selective downregulation of one type of TH response (here TH1) was due to selective anergy induction in TH1 cells thereby permitting an antagonistic TH2 response to prevail or, alternatively, whether it resulted from selective activation of TH2 responses which further inhibited TH1-mediated functions. In some cases, all mechanisms might play a role, but the relative significance of each, i.e. selective anergy, selective activation, and antagonistic regulation may be difficult to assess.

Many findings reviewed above can be accounted for by T cell anergy as a direct consequence of interactions between T cells and peptide-MHC complexes under conditions that fail to provide certain costimulatory signals. However, differential TH1/TH2 regulation may bridge two major mechanisms of peripheral tolerance, anergy and suppression. A particular method of antigen administration that preferentially induces anergy in- or selectively activates a subpopulation of T cells, may permit the outgrowth of those T cells which subsequently downregulate or suppress the antagonistic T cell subset. Under some conditions these regulatory functions of T cells may pay a more significant contribution to peripheral tolerance than anergy; and in some models, tolerance may largely or exclusively be induced by the action of regulator or suppressor cells.

1.2.4 iv The role of suppressor cells

Experimentally induced suppressor cells

The significance of active regulatory mechanisms mediated by suppressor cells has been studied in a variety of cell transfer systems, often with models of experimental autoimmune diseases, in which lymphocyte populations from tolerized donors were examined for their capacity to protect recipients from disease induction. In a murine model of thyroiditis, intravenous injection of the target antigen thyroglobulin (MTg) rendered mice unable to mount anti-MTg antibody responses upon subsequent challenge with MTg and LPS (for B cell activation). This state of tolerance could be transferred to naive recipients with splenocytes from tolerized donors. Selective depletion of lymphocyte subsets revealed that CD4+ T cells were responsible for tolerance and tolerance transfer in this model (Parish, Roitt, & Cooke, 1988). A role for suppressor cells was also shown for other strategies of tolerance induction such as oral administration of antigen. In these studies, resistance to experimental autoimmune diseases could be adoptively transferred with lymphocytes from donors that had been fed with the respective target antigens. For the Lewis rat model of EAE, the relevant
suppressor cells were CD8-positive (Lider et al., 1989) while protection from experimental autoimmune uveoretinitis could be transferred with lymphocytes from rats that had been fed with a peptide known to be recognized by CD4+ T cells (Gregerson, Obritsch, & Donoso, 1993).

Antigen-specific suppressor cells can also be induced when T cells encounter antigen in the presence of antibodies that block the CD4 or CD8 coreceptors. For a mouse model of transplantation tolerance Qin et al reported that donor CD4+ T cells tolerized with antigen (skin graft) and non-depleting CD4/CD8 antibodies not only prevented skin graft rejection in naive recipients but also endowed the recipient’s T cells with the capacity to transfer tolerance themselves to a second group of recipients, a phenomenon named “infectious tolerance” (Qin et al., 1993). The ability to provoke active suppressor mechanisms by targeting coreceptor with monoclonal antibodies was also shown for the mouse model of thyroiditis. (Hutchings et al., 1993).

**Evidence for naturally occurring regulator cells**

Several studies demonstrate that suppressor cells are not confined to tolerance induced by experimental manipulations but also appear to contribute to immune regulation under physiological conditions. Fowell and Mason showed that thymectomy and irradiation of a normally non-diabetic rat strain was sufficient to induce cell-mediated autoimmune diabetes. However, diabetes could be prevented by transferring T cells from syngeneic healthy donors, thus attesting the significance of cells within an intact immune system to control potentially autoreactive T cells. A detailed analysis of the T cell phenotype required to replenish the ability to resist diabetes revealed that only non-activated CD45 RC low CD4+ T cells were protective upon transfer. Although this phenotype applies to both memory T cells and recent thymic migrants, the latter were not required to transfer protection (Fowell & Mason, 1993).

Experimental autoimmune encephalomyelitis (EAE) represents an attractive model to study the role of regulator cells. In the acute model of EAE in the Lewis rat, a phase of inflammatory reactions in the CNS and limb paralysis is followed by spontaneous recovery after which animals are refractory to further disease induction. In rat and mouse models of EAE, protection from further phases of disease was found to be associated with post-recovery CD4+ T cells which upon transfer protected recipients against EAE (Welch, Holda, & Swanborg, 1980; Kumar & Sercarz, 1993). In vitro studies on these T suppressor cells showed that they proliferated in response to irradiated effector cells or a synthetic peptide
covering a likely effector TCR Vβ chain determinant. The mechanism as to how Ts cells downregulate effector cells in vivo has not been resolved. However, when stimulated with T cell effector determinants in vitro, these regulator cells were shown to release IL-4 (Karpus, Gould & Swanborg, 1992). In another study, their suppressive effect on IFNγ-production by T cells specific for the encephalitogenic antigen was inhabitable with antibodies to transforming growth factor-β (TGF-β), a cytokine known for its anti-inflammatory action (Karpus & Swanborg, 1991). Other experiments in which mice were depleted of CD8+ T cells (Jiang, Zhang & Pernis, 1992) or where the CD8 gene was non-functional (Koh et al, 1992) suggested that CD8+ T cells did not influence the recovery phase but conferred resistance to a second induction of disease. In another study, the transfer of an MBP-specific encephalitogenic cell line (Q4) directly after birth prior to the expression of MBP lead to a loss of resistance to repeated EAE induction in adults. In this Lewis rat model of EAE the resistance to a second challenge with activated Q4 cells was mediated by CD8+ T cells that proliferated in vitro in response to irradiated Q4. This population of CD8+ T cells was missing in mice neonatally treated with Q4 cells (Qin, Sun, & Wekerle, 1992).

In summary, selective pressures in the thymus and the periphery favour the inactivation of T cells forming high avidity TCR-MHC-peptide complexes by anergy and deletion. Different requirements for the activation of antagonistic T cell subsets and other regulatory or suppressor cells establish homeostatic control mechanisms which together with the inherent avidity and signalling threshold constraints on T cell activation serve to preserve the integrity of the immune system. However, the ability to induce autoimmune disease in experimental models and the occurrence of autoimmunity and allergies in humans demonstrate that this balance can be perturbed to an extent at which it can no longer re-adjust itself. The challenge to immunology is then to educate the immune system according to its own rules, to exploit the knowledge about mechanisms of peripheral tolerance to divert lymphocyte functions from erroneously attacking innocuous antigens into a normal state of tolerance.
1.3 BREAKING OF TOLERANCE AND IMMUNE INTERVENTION

1.3.1 How Peripheral Tolerance Can Be Broken

Various experimental manipulations enable T cells to break the rules that govern the maintenance of peripheral tolerance. A common protocol for priming TH1 type responses is to administer antigen subcutaneously in complete Freund's adjuvant (CFA), a mixture of oil and mycobacterial antigens mimicking a pathogenic insult. By this method, T cell mediated experimental autoimmune diseases can be induced with self antigen/CFA in susceptible animals. Antigen is retained at the site of injection and slowly released to draining lymph nodes where NK cells appear to initially set the scene by IFNγ production, presumably in response to the mycobacterial particles. This attracts and activates macrophages after which IL-2 producing T cells can be demonstrated by immunohistochemistry (Bogen, Fogelman, & Abbas, 1993). Autoreactive T cells are therefore driven to recognize antigen presented in the presence of costimulatory signals such as provided by activated macrophages within a lymphokine environment that favours inflammatory effector functions.

T cells which fell below the avidity threshold for negative selection in the thymus may nevertheless mediate high avidity interactions in the periphery under certain conditions. Immunization with a single peptide in CFA bypasses the need for antigen processing thereby enabling T cells to respond to subdominant or cryptic autoantigenic epitope. Significantly, peptide immunization also seems to drive immune responses to the whole protein to which animals initially failed to respond as shown for cytochrome c in the mouse (Mamula, 1993). Presumably, as a secondary effect to peptide priming, B cells specific for the cyt 81-104 peptide also bound and processed whole mouse cytochrome c and subsequently presented other cyt peptides. A similar observation was made in a mouse model of EAE where immunization with the disease-inducing agent myelin basic protein (MBP) initially only evoked T cell responses to the dominant N-terminal epitope of MBP (Acl-11) whereas after 40 days responses to subdominant were also evidenced, a phenomenon termed epitope spreading (Lehmann et al, 1992). Spreading of the immune response to subdominant and cryptic epitopes was also observed when the dominant epitope alone was used for priming, suggesting subsequent waves of priming by endogenous MBP.
As discussed for thymic selection, the TCR repertoire has to display some degree of degeneracy with respect to antigen specificity to allow for the recognition of antigens not presented during positive selection. For the H-2\textsuperscript{u}-restricted TCR specific for Ac1-11 of MBP, Gautam et al have shown that a polyalanine analogue of this peptide containing only five native residues was sufficient to activate a specific T cell hybridoma in vitro and to induce EAE in vivo (Gautam et al, 1992a). Wraith et al demonstrated in the same model that one of two peptide residues that serve as TCR determinants could be replaced by a number of alternative amino acids without abrogating TCR recognition (Wraith, Bruun, & Fairchild, 1992). A drawback of TCR crossreactivity is therefore the potential of self-reactive T cells to be activated by microbial or viral peptides that fulfil the same minimal requirements for TCR recognition as the self peptides. Furthermore, tissue injury may lead to the release and presentation of high amounts of previously sequestered antigens which, in combination with local inflammatory responses may suffice to activate autoreactive T cells.

The induction of autoimmune diseases with activated T cell clones bypasses the control mechanisms that might otherwise have prevented the induction of those cells. In a transgenic mouse model of EAE, some animals spontaneously developed autoimmune disease (Goverman et al, 1993). Presumably, in this situation the non-physiologically large number of self-reactive T cells defies most physiological control mechanisms.

1.3.2. Strategies For Immune Intervention

Autoimmunity, allergies, and organ transplant rejection represent areas for which the ultimate goal is to reverse detrimental immune responses towards tolerance to innocuous self and non-self antigens. Therapeutic approaches to ameliorate these conditions still rely heavily on the application of various non-specific immunosuppressive drugs (Thomson, Woo, & Cooper, 1992) which can be severely toxic and may compromise normal effector functions involved in immune surveillance and protective immunity (Bach, 1991). Other strategies in animal models aimed to prevent the access of autoreactive effector T cells to the target tissue by blocking surface molecules required for adhesion and transendothelial migration (reviewed in Cooke & Wraith, 1993). However, preventing the riot does not necessarily appease the aggressor. Thus, management of undesirable immune reactions would be greatly improved if T cells could be targetted more specifically. In models of EAE with restricted
effector TCR chain usage, attempts have been made to induce regulator cells against effector T cells by TCR vaccination. As mentioned before, such regulator or suppressor cells may be involved in recovery from and further resistance to acute forms of EAE. However, while TCR vaccination conferred protection in one study (Gaur et al, 1993), it enhanced disease in other cases (Desquenne-Clark et al, 1991). Another approach has been the blockade of MHC molecules with peptides that associate with the same restriction element as the target peptide thereby competing for binding and preventing ligation with the self-reactive TCR. (Gautam et al, 1992b; Hurtenbach et al, 1993). A common drawback in those systems is, however, that blocking is not permanent and may require continuous treatment.

The knowledge of antagonistic TH1/TH2 functions and its control by distinct sets of cytokines has encouraged attempts to selectively block or enhance immune responses by the administration of cytokines, anti-cytokine antibodies or soluble cytokine receptors (reviewed in Cooke & Wraith, 1993; Lanzavecchia, 1993). While this strategy was successful in some models, it may prove difficult where tissues are not easily accessible by soluble therapeutic agents or in situations where cytokine regulation does not fall into clear-cut TH1/TH2 categories.

The most specific strategy for immune intervention is to target the TCR with its own ligand, the MHC-peptide complex. One approach suggested by a number of recent studies could involve alterations of critical peptide residues in such a way that it engages in the trimolecular MHC-peptide-TCR complex without eliciting T cell responses (antagonism) or where the altered peptide ligand triggers some but not all signals required for T cell activation (partial agonism). These observations revealed that ligation of the TCR by peptide-MHC is not an all- or- none event but that different intracellular signalling pathways connected to the TCR/CD3 and CD4- or CD8 complex can be triggered selectively depending on the nature of the peptide ligand (reviewed in Fairchild et al, 1994; Sette et al, 1994). Furthermore (and somewhat homologous to the significance of coreceptor-mediated costimulatory signals), a partial agonist may not only fail to activate a T cell but render it anergic to subsequent stimulation with the natural ligand (Sloan-Lancaster, Evavold, & Allen, 1993).

The application of peptide antagonists or partial agonists requires in depth knowledge of critical residues and relevant substitutions. However, the numerous strategies to induce peripheral tolerance outlined earlier clearly reveal that T cell responses can also be downregulated by their natural ligand under appropriate conditions. Generally, in those studies, soluble protein antigens injected intravenously or intraperitoneally induced T cell nonresponsiveness.
Antigen administered in incomplete Freund's adjuvant (IFA) that lacks mycobacterial particles and therefore does not provoke inflammatory responses, may also induce specific T cell tolerance (Smilek et al, 1991; Gaur et al, 1992).

While these approaches employ somewhat artificial routes of antigen administration, antigen uptake through mucosal surfaces represents a major natural route by which the immune system is constantly challenged with foreign antigens. Some of these antigens may be detrimental, but the majority is innocuous. In the case of food proteins that pass the mucosa of the small intestine, antigens provide essential metabolites against which aggressive immune responses would be devastating. Local inflammatory allergic responses to certain food substances or inhaled antigens are the exception to the rule that antigens encountered via mucosal surfaces are generally tolerized by the immune system. Oral tolerance, the phenomenon of specific immunological unresponsiveness induced by feeding antigen, has been known for a long time and studied with dietary proteins (reviewed by Mowat, 1987) as well as potential self-antigens (reviewed by Thompson & Staines, 1990). More recently, antigen inhalation was also shown to downregulate specific immune responses (reviewed in Holt & McMenamin, 1989).

The prospect of antigen-specific immune intervention by natural routes is intriguing and has already encouraged clinical trials for oral tolerance in human autoimmune or inflammatory conditions (Weiner et al, 1993; Trentham et al, 1993). However, a thorough understanding of the mechanisms that mediate immunological tolerance to antigens taken up through the gut and the respiratory tract not only provide a close insight into immune regulation in general; it is also a prerequisite for safe and successful clinical applications. This thesis aims to contribute to the understanding of oral tolerance and to examine the potential to inhibit T cell-mediated autoimmune responses by antigen administered through the respiratory tract. In both situations, antigen is confronted with lymphoid cells and structures which are peculiar to the gut or the respiratory tract. However, both routes are also sites where antigen may be efficiently absorbed into the systemic circulation. When considering phenomena of oral- and inhalation tolerance the question therefore arises as to which extent the impact of fed or inhaled antigens on systemic immune responses can be accounted for by mechanisms of peripheral tolerance in general (such as induced by the intravenous or intraperitoneal route) and to which extent by regulatory mechanisms that are unique to mucosal-associated lymphoid elements. Furthermore, the lymphoid tissue associated with the small intestine and the respiratory tract bear both striking similarities and obvious differences. It will therefore be interesting to compare the impact on immune responses by antigen
administered through both routes. Before the issues addressed in this thesis are introduced in more detail, an overview of the lymphoid tissues and cells in the small intestine and the respiratory tract is included to aid further discussion.

1.4 LYMPHOID TISSUES AND ANTIGEN HANDLING IN THE SMALL INTESTINE AND THE RESPIRATORY TRACT

1.4.1 Histologic Organisation And Cell Types Of The Small Intestine

The lining of the intestinal wall is thrown into large elevations (plicae circularis) that include mucosa and submucosa. The surface is studded with fingershaped mucosal projections, the villi. These contain a core of highly cellular, loose connective tissue, the lamina propria, covered by a simple columnar epithelium. The intestinal glands or crypts of Lieberkuhn form simple tubular structures with pore-like openings between the bases of the villi (Rhodin,1974; Weiss & Greep, 1977).

1.4.1 i Villi and crypts

The absorptive cell with its highly differentiated structure (microvilli) is characteristic of the villi whereas within the crypts, relatively undifferentiated, frequently dividing columnar cells prevail. Other cell types include: goblet cells which produce the pre-epithelial mucus. Several types of endocrine cells produce hormones including serotonin, gastrin, secretin (a glucagon-like hormone), and possibly noradrenalin and cholecystokinin (reviewed in O’Dorisio, 1986; Freier & Lebenthal, 1989). Paneth cells in the crypts secrete peptidases and lysozyme. M (microfold) cells are characteristic of the epithelial area covering Peyer’s Patches and are probably specialized in antigen sampling. Intraepithelial lymphocytes are mainly T cells of which 80-90% are CD8+ (reviewed in Pabst, 1987).

1.4.1 ii Lamina propria

The lamina propria contains large numbers of lymphocytes, granulocytes, macrophages, and plasma cells. The majority of lymphocytes are CD4+ T cells. Mature small B cells are relatively rare, but plasma cells are present in large numbers, most of which are IgA positive (80%) followed by IgM+ and IgG+ B cells (reviewed in Pabst, 1987).
1.4.1 Peyer's Patches (PP)

PP constitute the lymphoid organ of the intestinal wall. They are covered by a distinct PP-associated dome epithelium ("dome", because the follicles cause the epithelium to bulge into the lumen like the vault of a church). This dome epithelium is devoid of the crypt/villi structure. M cells are distributed evenly over the higher regions of the dome representing about 15% of the total cell population. The absence of goblet cells renders the dome area a mucus-free zone and therefore a weak point in the intestinal barrier. Of great functional importance is the ability of M cells to transport large molecules and particles as shown for India ink, horse radish peroxidase and others. M cells transport but do not seem to degrade antigen. The basement membrane of the dome shows a typical porosity, thereby enabling macrophages and other cells from the lamina propria to interact with M cells, possibly by taking up particles and molecules transported by M cells.

PP consist of four compartments defined by different structural elements and functions: upon antigenic stimulation germinal centres comprising the B cell areas develop within the lymphoid follicles. Most B lymphocytes differentiate into IgA plasma cells. Processes of I-A-positive follicular dendritic cells are prominent in germinal centres, and some T cells are also present. Small mature (mainly B) lymphocytes surround the follicles forming the corona. Specialized venules with a high cobble stone type epithelium are characteristic of the interfollicular area. Lymphocytes emigrate from the blood and enter the PP via these postcapillary venules (PCV). Migration patterns of lymphocytes are directed by homing receptors, and some lymphocytes specifically home to the gut. The interfollicular area contains mainly T lymphocytes, the majority of them belonging to the CD4 positive lineage. Processes of I-A-positive dendritic cells are found throughout this area, sending branches between T cells. The dome area underneath the epithelium contains a mixture of B-and T lymphocytes, plasma cells and macrophages (reviewed in Pabst, 1987; Kawanishi, 1989; Nagura, 1988).

1.4.2 Antigen Presentation

The apparently intimate contact between dendritic cells and M cells in the interfollicular area suggests that dendritic cells may take up antigen transported by M cells and present it to T cells in the interfollicular area. Within germinal
centres of PP, I-A positive follicular dendritic cells trap and present antigen to B cells (reviewed in Kawanishi, 1989; Nagura, 1988).

It has been reported that IFNγ-induced I-A-positive gut epithelial cells can replace macrophages or dendritic cells as APC in mixed lymphocyte reactions (Mayer & Shlien, 1987). In these experiments, the epithelial cells seemed to selectively stimulate CD8+ T cells. Since a high proportion of intraepithelial cells are CD8+, it is conceivable that they might also be relevant as APC in vivo. In another study, intestinal epithelial cells were found to inhibit in vitro lymphocyte proliferation, an effect which was reversible with a blocker of prostaglandin synthesis or exogenous IL-2 (Santos et al, 1990).

1.4.3 Lymphocyte Trafficking

Two pools of small recirculating lymphocytes have been described, one migrating mostly through peripheral lymph nodes and others mainly through gut-associated lymphoid tissue (GALT). Lymphocytes which derived from the gut or mesenteric lymph nodes showed a preference for migration to other organs lined with mucous membranes such as the bronchial tract, salivary-, lacrimal- and mammary glands. These observations gave rise to the concept of mucosa-associated lymphoid tissue (MALT).

PP lack an afferent lymph supply so that lymphocytes may only enter PP from the mucosal circulation via PCV. Antigen-primed lymphocytes exit through the lymphatics to mesenteric lymph nodes, the thoracic duct, spleen, and finally lodge in the lamina propria. Alternatively, some lymphocytes may home directly from the mesenteric lymph nodes to PP without entering the thoracic duct and passing through the spleen (reviewed in Kawanishi, 1989).

1.4.4 The Gut Epithelium- An Extrathymic Place Of T Cell Differentiation

Recent studies revealed a function of the gut epithelium as a thymus-independent site of T cell development. Phenotype analysis of intraepithelial lymphocytes (IEL) showed a distribution of 10% CD4+TCRαβ+ cells and 80% CD8+ T cells which bear either TCRαβ (70%) or TCRγδ (30%) chains. Some of the TCRαβCD8+ cells and all of the TCRγδ cells expressed homodimeric CD8 molecules composed of two alpha chains instead of the αβ heterodimer.
Homodimeric CD8αα⁺ T cells were not detectable in any other peripheral lymphoid tissues or the thymus (Guy-Grand et al., 1991; Rocha, Vassalli, & Guy-Grand, 1991). The development of these TCRγδ cells (which were either double negative or express CD8αα) was independent of the thymus and also followed different rules for development. In MHC class I or class II knockout mice, TCRαβ IEL were missing while the population of TCRγδ⁺ cells was even increased, suggesting that classical MHC molecules were not involved in the selection of TCRγδ⁺ cells (Schleussner & Ceredig, 1993). Disrupting the gene for the γ chain abrogated normal development of T cells in the thymus. Among IEL, all thymus dependent T cells were missing while the development of all TCRγδ cells and the small population of TCRαβ⁺CD4⁺CD8β⁻ cells was unaffected (Malissen et al., 1993). Furthermore, thymic dependent T cells specific for a Mls superantigen or the male-specific H-Y antigen were deleted in the thymus in animal expressing these antigens while, in contrast, the antigen appeared to be required for the maturation of thymus independent IEL. In female mice, all H-Y-specific thymus independent cells were double negative and lacked granules while in male mice the same population expressed high levels of TCR and CD8. Although these T cells could mediate primary cytotoxic responses in vitro, there were no lesions or other signs of autoimmune reactions in vivo (Rocha, Vassalli, & Guy-Grand, 1991; Rocha, von Boehmer, & Guy-Grand, 1992).

The antigens recognized by TCRγδ cells have not been clearly defined. However, in agreement with γδ T cell selection in the absence of classical MHC molecules, antigen recognition by mature γδ T cells was also shown to be independent of MHC class I or II molecules. Disrupting conventional MHC-antigen presentation had no effect on the stimulation of γδ T cells while abrogating all TCRαβ cell-mediated responses in vitro. Instead, antigen recognition by a γδ T cell line correlated with the expression of the nonclassical MHC molecule TL (Schild et al., 1994). The authors suggest that the structural similarities between immunoglobulins and γδ TCR imply similar modes of antigen recognition independent of specialized APC.

1.4.5 Structure Of The Respiratory Tract

The respiratory system consists of portions anterior and posterior to the pharynx where respiratory and digestive tracts cross. The anterior respiratory tract includes nostrils, nasal cavity, and pharyngeal tract. The posterior tract consists of larynx, trachea, bronchi, and lungs. The trachea extends from the
narrow end of the larynx into the thoracic cavity where it branches into left and right bronchi. Large primary bronchi lead into the left and right lung, where they branch repeatedly, diminishing in size with each division. As the tubes become smaller their walls become thinner. The terminal bronchioles branch into several alveolar ducts leading into alveolar sacs, each composed of several alveoli. These structures have very thin walls invested with fine close-meshed networks of large thin-walled capillaries (reviewed in Hummel, Richardson, & Fekete, 1966).

1.4.6 Routes followed by Antigen In The Respiratory Tract

1.4.6 i Role of particle size

A number of mechanisms protect the lung from inhaled antigenic material. In the upper respiratory tract, the first barrier is a layer of mucous and the action of cilia. The deposition of inhaled particles within the respiratory tract depends to a large extent on their size. Several studies suggested that the majority of droplet particles greater than 12\(\mu\)m in diameter were retained in the nose, whereas those of 4\(\mu\)m in diameter were deposited in the nose, bronchi and bronchioles. Smaller particles of 0.8-1.6 \(\mu\)m in diameter were partly retained in the alveoli or breathed out again (reviewed in Yoffey & Courtice, 1970).

1.4.6 ii Absorption into lymphatics and circulation

Within the nasopharynx, the submucosa underlying the epithelium is rich in lymphatic vessels from which the lymph drains into the deep cervical lymphatics. To ascertain the pathway of antigenic material, substances of varying molecular weight and size were applied to the nasal mucosa. Small molecules such as water and some salts or dyes were mainly absorbed into blood capillaries and to a lesser extent into lymphatic vessels. Dyes such as trypan blue and Evans blue with a molecular weight of about 1000 rapidly entered the cervical lymph when applied in solution to the nasal mucosa. In several studies intranasally administered molecules up to the size of ovalbumin passed into the lymph (reviewed in Yoffey & Courtice, 1970). When fluorescent-or radiolabelled bovine serum albumin (BSA) was administered as an aerosol with droplet sizes around 2\(\mu\)m or 7 \(\mu\)m in diameter, fluorescent deposits lined the whole of the respiratory tract of rabbits, including finely dispersed granules in alveolar ducts and sacs (Willoughby & Willoughby, 1977). Kinetic experiments with radiolabelled BSA in the same study demonstrated that the majority of the
label (about 70%) was found in the stomach and subsequently in the serum and urine. At any time, even after repeated aerosol exposure, the proportion of total label in the lung did not exceed 4% suggesting that there was no pulmonary sequestration of soluble protein. When BSA was administered directly through the nostrils rather than in an aerosolized form, it also entered the circulation, apparently as intact molecules rather than degradation (non-TCA precipitable) products.

Soluble proteins thus appear to be readily absorbed into the rich network of lymphatic-and blood vessels surrounding the whole of the respiratory tract. In contrast, studies with carbon particles and fluorescent microspheres showed that particulate antigens did not readily penetrate the mucosa and epithelium. Instead, the alveolar macrophage (AM) as the prominent cell in the lower respiratory tract phagocytosed particles and carried them to the draining (tracheobranchial) lymph nodes (Harmsen et al, 1985). In contrast to their efficient handling of particulate antigen, AM were defective in stimulating specific in vitro T cell proliferative responses to soluble antigens, in striking contrast to splenic and peritoneal macrophages (Ullrich & Herscowitz, 1980). While this study suggested an inherent difference in antigen handling by AM rather than active suppression, it has also been reported in support of AM-mediated inhibition that lymphocyte responses to mitogens were substantially improved after depletion of AM (reviewed in Lawrence, 1988). Recent work by Holt et al showed that the inhibitory effect of AM on mitogen-induced T cell proliferation could be abrogated by granulocyte/macrophage colony-stimulating factor, GM-CSF (Bilyk & Holt, 1993).

1.4.7 Lymphoid Tissues

Antigen in the respiratory tract may be drained to several lymph nodes. In the mouse, these are the superficial- and deep cervicalys and the mediastinal lymph nodes (reviewed in Hummel, Richardson, & Fekete, 1966).

The bronchus- associated lymphoid tissue (BALT) is morphologically very similar to Peyer's Patches (PP) of GALT (Bienenstock, Johnston, & Perey, 1973). It lies within the lamina propria of large bronchi near bifurcations. BALT is covered by a specialized flattened epithelium which, like M cells of PP, is devoid of mucous and cilia. There is evidence that these specialized epithelial M cells can pinocytose and transport soluble and particulate antigens across the bronchial mucosa. Similar epithelial cells also cover smaller lymphoid
aggregated in the more distal bronchioles. However, unlike in PP, no prominent germinal centres were identified in BALT (reviewed in Gil & Daniele, 1988). The cell population in BALT contains both T and B cells although the relative proportions of both types of lymphocytes vary among different reports (reviewed in Lawrence, 1988). By microscopy, the epithelium overlying BALT was heavily infiltrated with lymphocytes, but only few plasma cells were detected (Bienenstock, Johnston, & Perey, 1973). When the cellular components of bronchial lavage were analyzed, the lung parenchyma also contained lymphocytes of which a high proportion (about 65%) were T cells.

1.5 DOWNREGULATION OF SYSTEMIC IMMUNE RESPONSES BY ORAL AND INTRANASAL ANTIGEN ADMINISTRATION

1.5.1 Oral Tolerance

The term “oral tolerance” describes the state of specific immunological nonresponsiveness induced by oral or intragastric administration of antigen. The phenomenon of oral tolerance has been known for a long time. Wells showed in 1911 that feeding guinea pigs rendered them refractory to anaphylactic reactions against dietary proteins (Wells, 1911). For the last two decades, Ovalbumin (OVA) has served as a popular model protein for numerous studies addressing the mechanism of oral tolerance (reviewed in Mowat, 1987). The potential of orally administered antigens to downregulate systemic immune responses has also been widely explored with target antigens in experimental models of autoimmune inflammatory diseases such as experimental autoimmune uveoretinitis (EAU), arthritis (EAA), and encephalomyelitis (EAE, reviewed in Thompson & Staines, 1990; Weiner et al, 1994). Oral tolerance was shown to affect T cell mediated effector functions such as DTH and pathological signs of inflammatory reactions. Antibody levels were also reduced, although hapten-carrier experiments suggested that this was due to a lack of T cell help rather than by direct B cell tolerance (Hanson et al, 1977; Richman et al, 1978; Titus & Chiller, 1981).

In many models, two major mechanisms, T cell mediated suppression and anergy have both been implicated in oral tolerance. In the OVA mouse model, numerous reports showed that tolerance was transferrable to naive recipients with splenocytes from OVA-fed donors (Miller & Hanson, 1979; Mowat, 1986; Lamont et al, 1988). Adoptive transfer of tolerance was also achieved with CD8+
T cells from Peyer's patches (Ishii et al, 1993). Transfer of tolerance was not confined to cellular elements but was also demonstrated with serum collected from donors one hour after OVA feeding (Strobel et al, 1983; Peng, Turner & Strobel, 1990). However, when T cell responses such as proliferation and IL-2 production were examined in vitro, there was no evidence for suppression. Rather, the results were consistent with T cell anergy (Richman et al, 1978; Melamed & Friedman, 1993). Oral tolerance in the Lewis rat model of EAE has been induced by feeding the target protein myelin basic protein (MBP). The suppressive effect of oral antigen administration in this model was attributed to the anti-inflammatory cytokine transforming growth factor-β (TGF-β) released by CD8+ T cells upon stimulation with the same antigen that had previously been fed. The release of TGF-β appeared to be inducible by a non-encephalitogenic but not by the encephalitogenic MBP fragment. In these studies the action of CD8+ T suppressor cells was also assessed by the ability of TGF-β to non-specifically reduce immune responses to a second antigen in a bystander fashion (Miller, Lider & Weiner, 1991; Miller et al, 1992a). However, in other studies on the same model, albeit under different experimental conditions, there was no evidence for suppression upon cell transfer whereas diminished in vitro T cell responses from MBP-fed rats were consistent with T cell anergy (Whitacre et al, 1991). Studies on oral tolerance in a Lewis rat model of EAU emphasized the dose dependence of fed antigen to induce modes of oral tolerance that were consistent with either suppression of anergy. While low dose feeding revealed hallmarks of suppression such as the ability to adoptively transfer tolerance and to induce bystander inhibition to other (not previously fed) epitopes, high dose feeding appeared to induce a strictly epitope-specific and non-transferrable form of tolerance. The mechanisms of suppression in this model were not resolved. However, the finding that a disease -inducing peptide known to activate CD4+ T cells was able to induce suppression suggested that the suppressor cells belonged to the CD4 rather than the CD8 lineage (Gregerson, Obritsch, & Donoso, 1993). The dose of intragastrically applied antigen may not only dictate the mechanism of oral tolerance but also decide between tolerance induction versus priming of an immune response. In the OVA mouse model, different oral doses of OVA induced either tolerance or supported T cell priming while some amounts of fed protein had no apparent effect on a subsequent challenge (Lamont, Mowat, & Parrott, 1989). These findings therefore suggest that the intragastric route of antigen administration does not necessarily provide a default mechanisms for tolerance induction. The evidence from various models of oral tolerance also reveals a broad spectrum rather than a universal mechanisms of tolerance induced by feeding antigen.
1.5.2 Downregulation Of Immune Responses By Protein Inhalation

While oral tolerance boasts a long history of investigation, antigen inhalation represents a more recent approach to modulate systemic immune responses in an antigen-specific manner. The problems faced by the immune system are, however, very similar. Like the gastrointestinal tract, the respiratory tract is in constant contact with numerous antigenic particles of which only a minority is potentially pathogenic. The similarities between GALT and BALT suggest that the task of selectively attacking potentially detrimental while sparing innocuous agents might be solved in a similar fashion. Since IgE-mediated activation of mast cells constitutes the major hazard in allergies to airborne particles, a series of studies focussed on aerosol-induced tolerance with emphasis on the IgE isotype (reviewed in Holt & McMenamin, 1989). A recent report showed that exposure of rats to aerosolized OVA induced a complex mechanism of tolerance whereby IFNγ producing CD8+ T cells abrogated a transient CD4+-mediated OVA-specific IgE response. This was interpreted as a CD8+ T cell mediated deviation from a CD4+ T cell -induced TH2 response. In this model the tolerant state was transferrable to naive animals with CD8+ T cells (McMenamin & Holt, 1993). Other studies addressed the role of APC's in the induction of inhalation tolerance. Early reports already mentioned the inhibitory effect of alveolar macrophages (AM) on T cell proliferation (reviewed in Lawrence, 1988). More recently, the immunosuppressive capacity of AM was shown to be abrogated by GM-CSF (Bilyk & Holt, 1993). Furthermore, AMs also appeared to suppress the ability of pulmonary dendritic cells to activate T cells (Holt et al, 1993). In a mouse model of allergy to house dust mite antigens, inhalation of a single peptide representing a dominant CD4+ T cell epitope diminished in vitro IL-2 production as well as in vitro antibody secretion by spleen cells from primed mice, suggesting a CD8+ T cell-independent mechanism of suppression (Hoyne et al, 1993). However, antigen inhalation has not yet been widely investigated as a strategy for tolerance induction in inflammatory autoimmune diseases. One report mentioned the amelioration of EAE by MBP inhalation in Lewis rats, but no data was provided or referred to (Weiner et al, 1991). One study demonstrated that nasal administration of retinal antigens suppressed EAU in Lewis rats (Dick et al, 1993). To our knowledge, this has been the only evidence that antigen inhalation could abrogate inflammatory autoimmune responses apart from the work presented in this thesis.
1.5.3 Aim Of This Thesis

This project explores antigen administration via the gastrointestinal- and the respiratory tract as a potential means to specifically downregulate systemic T cell-dependent immune responses to soluble antigens. Previously published work has provided evidence for both anergy and suppression. In an attempt to distinguish between these two mechanisms, the experiments presented in this thesis concentrate on the significance of individual epitopes. An engagement of CD8+ T suppressor cells implies that distinct epitopes are required for suppression (MHC class I-restricted) of a MHC class II-restricted immune response. As shown for the Lewis rat model of EAE, only a non-encephalitogenic peptide could induce CD8+ T cells to release TGF-β (Miller et al., 1993). A MHC class II restricted epitopes may either induce CD4+ T cell-mediated regulation or more directly T cell anergy. In the first case, we may be able to demonstrate bystander suppression if CD4+ Ts cells also operate by the release of inhibitory (possibly TH2 type) lymphokines. In the latter case we expect a strictly epitope-specific phenomenon which presumably requires relatively high doses of fed or inhaled antigen. Furthermore, if our observations are consistent with anergy rather than suppression, then, similarly to the HEL model of peripheral tolerance, we may be able to demonstrate a higher degree of downregulation if the overall avidity of TCR-peptide-MHC interactions is increased. This project features experiments on tolerance induction in two models, the H-2d mouse model of oral tolerance to OVA and the H-2u mouse model of EAE.

1.5.3 i Oral tolerance to OVA

Previous work addressed the effects of orally administered whole OVA on T cell-mediated immune responses upon challenge with the intact OVA molecule. The OVA peptide 323-339 was identified as a major epitope for I-A<sup>d</sup>-restricted CD4+ OVA-specific T cells (Shimonkevitz et al., 1984). For this thesis, OVA 323-339 is used as a tool to investigate in some more detail which epitopes of OVA are effective and which epitopes are affected. If the induction of CD8+ T suppressor cells is a prerequisite for oral tolerance to OVA, then oral administration of OVA but not OVA 323-339 will be effective. Furthermore, CD8+ T cells induced by antigen feeding may have to encounter their specific epitope again at the time of immunization in which case feeding OVA would
downregulate immune responses after priming with OVA but not with OVA 323-339.

In the light of reports emphasizing the role of B cells as an APC to either anergize naive T cells or deviate an immune response towards the TH2 spectrum, the PP and the spleen as rich sources of B cells may represent lymphoid organs for the preferential downregulation of food antigen-specific TH1 responses, either by direct anergy or TH2 deviation. This possibility will be addressed by an isotype-specific analysis of OVA-specific IgG. Furthermore, if TH2 cells play a role, they may be considered as suppressor cells for TH1 responses with the potential to downregulate responses to a second antigen in a bystander fashion.

The ability to transfer tolerance with serum from OVA-fed mice revealed a peripheral component which -at least in recipients- operates independently of the gut. Apart from a yet unidentified tolerogenic factor, whole immunogenic OVA which resembled the native form of the molecule was also detectable in the serum (Peng, Turner, & Strobel, 1990). This project includes experiments to examine both the tolerogenic and antigenic properties of serum from OVA-fed mice.

1.5.3 ii Studies on oral-and inhalation tolerance in the H-2u mouse model of EAE

Experimental autoimmune encephalomyelitis (EAE) represents an instructive model for acute inflammatory autoimmune disease, and in certain cases displays a chronic-relapsing course similar to multiple sclerosis in humans. Although originally induced with whole brain and spinal cord material, it became clear that CD4+ T cell dependent immune reactions directed against myelin proteins played a critical role. In many models of EAE, myelin basic protein (MBP) and proteolipid protein (PLP) are the major target antigens. EAE is inducible by immunizing susceptible animals with myelin antigens in CFA or by directly transferring specifically activated CD4+ T cells (reviewed in Wraith, 1992). In the H-2u mouse model of EAE the dominant I-Au-restricted T cell response is directed to the acetylated N-terminal nonapeptide of MBP (Ac1-9) which on its own is sufficient to induce EAE (Zamvil et al, 1986). The interactions of this peptide both with its specific TCR and with the I-Au restriction element have been analyzed in detail (Wraith et al, 1989; Wraith, Bruun, & Fairchild, 1992). Glutamine at position 3 and proline at position 6 serve as determinants for TCR recognition, whereas lysine 4 and arginine 5 are involved in binding to the I-Au molecule (Gautam et al, 1992a). In previous studies, analogues of Ac1-9 (or Ac1-11) with alanine or tyrosine at position 4 were shown to bind to the I-Au
molecule with higher affinity than the original peptide with lysine at position 4. Although competitive binding studies revealed an at least 10-fold higher affinity of 4A over 4K for I-A\textsuperscript{u}, only the 4Y analogue remained stably bound to I-A\textsuperscript{u} for at least 4 hours (Wraith et al, 1989; Fairchild et al, 1993). A recent report features a model of Ac1-9 complexed to the peptide binding groove of the I-A\textsuperscript{u} molecule, showing that the phenyl ring of tyrosine fits optimally into a single predominant hydrophobic pocket within the binding groove (Fairchild et al, 1994). Thus, a hierarchy of MHC binding affinity was defined as Ac1-11 (or Ac1-9) [4Y]>>[4A]>>[4K]. We have applied this well characterized MBP epitope to study the effect of peptide feeding and inhalation on the course of EAE. This model permits the modulation of the MHC-peptide-TCR avidity based on a hierarchy of affinity. Therefore, if direct MHC-peptide-TCR interactions are critical, then the higher affinity analogues of Ac1-9 may be more potent tolerogens when administered intragastrically or through the respiratory tract. Furthermore, although Ac1-9 is the dominant encephalitogenic epitope in this model, the I-E\textsuperscript{u}-restricted peptide 35-47 of MBP represents a subdominant epitope upon immunization with whole MBP (Zamvil et al, 1988). This peptide will be used to address the significance of epitope dominance for the induction of tolerance.
## CHAPTER 2

Materials And Methods

### 2.1 LIST OF MATERIALS AND SUPPLIERS

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
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</thead>
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<tr>
<td>acetone</td>
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</tr>
<tr>
<td>adjuvant complete Freund (CFA)</td>
<td>DIFCO, Detroit Michigan, USA</td>
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</tr>
<tr>
<td>albumin, chicken egg (ovalbumin)</td>
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<td>grade V</td>
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</tr>
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<td>BDH, Poole England</td>
</tr>
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<td>ammonium sulphate</td>
<td>Novabiochem, Nottingham, UK</td>
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<td>Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOB)</td>
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</tr>
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</tr>
<tr>
<td>goat anti-mouse IgM (µ-chain specific), peroxidase conjugate</td>
<td>Sigma, Poole, Dorset, England</td>
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hydrochloric acid (HCl), 36% w/w
hydroxybenzotriazole (HOBT)
Iscove's Modified Dulbecco's Medium (IMDM)
L-amino acids for peptide synthesis
Liquid scintillation cocktail OptiPhase 'HiSafe2'

M. tuberculosis H37 RA
methanol
Novachrome 600 dye
NovaSyn KA resin
NovaSyn KR resin
o-phenylenediamine dihydrochloride (ODP)
pertussis toxin
phenol
phosphate buffered saline (PBS)
Dulbecco's w/o calcium and magnesium, w/o sodium bicarbonate
piperidine

polyoxyethylene-sorbitan monolaurate (Tween 20)
propanol

rat anti-mouse IgG1: Biotin
sample bags, for use with 1205 Betaplate
streptavidin-horseradish peroxidase
sulphuric acid, about 98%
trifluoroacetic acid (TFA)

trypan blue
trypsin inhibitor, from soybean
trypsin- chymotrypsin inhibitor, from soybean
tuberculin PPD, bovine

[Methyl-³H] Thymidine

May & Baker LTD, Dagenham, England
Novabiochem, Nottingham, UK
GIBCO BRL, Paisley, Scotland

Novabiochem, Nottingham, UK
Wallac, Turku, Finland
made by Fisons, Loughborough, England

DIFCO, Detroit Michigan, USA
Fisons, Loughborough, England
Novabiochem, Nottingham, UK
Novabiochem, Nottingham, UK
Novabiochem, Nottingham, UK
SIGMA, Poole, Dorset, England

Porton products, Salisbury, UK
BDH, Poole England
GIBCO BRL, Paisley, Scotland

Aldrich Chemical Co LTD,
Gillingham, Dorset, England
SIGMA, Poole, Dorset, England

Hayman Limited, Whitham, Essex, England
serotec, Kidlington, Oxford, England
WALLAC, Oy Turku, Finland

Amersham, Little Chalfont, UK
BDH, Poole England
Aldrich Chemical Co LTD,
Gillingham, Dorset, England
SIGMA, Poole, Dorset, England
SIGMA, Poole, Dorset, England
SIGMA, Poole, Dorset, England

Central Veterinary Laboratory,
Weybridge, England
Amersham, Little Chalfont, UK
2.2 PREPARATION OF ANTIGENS

2.2.1 Purification Of Myelin Basic Protein Form Pig Spinal Cords (Pig MBP)

MBP was prepared from pig spinal cords according to the procedures of Eylar, Kniskern, & Jackson (Eylar, Kniskern, & Jackson, 1974). Spinal cords were obtained from a local slaughter house and either immediately used or kept at -80 °C. For all procedures only ice cold reagents were used. Samples were centrifuged at 4 °C and handled in the cold wherever possible. No protease inhibitors were added.

For each preparation of MBP, 50 g pig spinal cord was homogenized with 125 ml methanol in a Servall Omni-mixer. This homogenate was thoroughly mixed with 250 ml chloroform and poured into a separating funnel. When the two phases were well separated, the lower lipid extract was discarded while the upper phase was mixed with diatomaceous earth to give a rather solid mud pie. This mixture was placed in a Buchner funnel on filter paper (Whatman) and extensively washed with acetone (approximately 500 ml) to extract residual lipids. As much acetone as possible was removed by vacuum pumping after which the earth was distributed as a thin layer on a piece of foil and left to dry completely. The dry powder was mixed with 500 ml H₂O.HCL pH2.1, and stirred for approximately 16 hours (overnight). The slurry was filtered to yield a clear solution. The pH was raised to 5.5 with NH₄OH and the cloudy sample stirred for 1 hour. The precipitate was spun at 15,000 x g for 20 min in a Sorvall RC-5B Superspeed centrifuge (DuPont). To the clear supernatant (typically 300-400 ml) 354g/l ammonium sulphate were slowly added. This corresponded to 50% saturation at 4°C (Dawson et al, eds., 1969). When all the ammonium sulphate was completely dissolved, the pH was adjusted to 6.0 with NH₄OH, and the sample left stirring for about 16 hours (overnight). The precipitate was spun for 30 min at 15,000 x g and the supernatant discarded. The pellet was dissolved in 15 ml H₂O. To this solution, 135 ml acetone and 135 µl 12 N HCL were added, and the mixture stirred for 1 hour. The precipitate was spun at 15,000 x g for 30 min. The pellet was dried on an oil vacuum pump, and then left in a fume cupboard until the acetone was fully evaporated. The dried pellet was resuspended in 20 ml H₂O and dialyzed 2x for 1 day each against 1.5-2 l H₂O. The sample was frozen on liquid nitrogen and lyophilized on a high vacuum pump (EDWARDS) for approximately 30 hours. The yield of MBP from each
preparation was about 50-100 mg lyophilized powder. The purity of MBP was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) under reducing conditions, and Coomassie blue staining of gels. The MBP preparations (20 µg loaded per lane) showed a single predominant band at 18.5 kD, corresponding to the previously reported molecular weight of the porcine MBP isotype (reviewed in Fritz & Mc Farlin, 1989). A few (4-5) faint bands of lower molecular weight probably represented small amounts of proteolytic degradation products of MBP (not shown). The identity of the purified protein with MBP was confirmed by Western blotting (not shown) using the monoclonal antibody clone 12 (kindly provided by Nigel Groome, Oxford) that was shown to recognize the epitope 82-87 of intact MBP from a variety of species (Hruby et al, 1987).

2.2.2 Preparation Of Mouse Spinal Cord Homogenate

Spinal cords were flushed from spines with the help of a water-filled syringe. The freshly isolated cords obtained from various strains of inbred mice were homogenized with a Dounce homogenizer, frozen at -80 °C and lyophilized. The lyophilized powder was stored at 4 °C.

2.2.3 Serum Samples

In chapter 3, sera from OVA-fed mice were used as a source of antigen for in vivo transfer experiments, and in vitro lymphocyte proliferation assays. Balb/c mice were fed 20 mg OVA in 200 µl PBS or 200 µl PBS alone. After 1 hour, mice were deeply anaesthetized with ether and bled from the neck vein. Blood samples were left to clot at room temperature for approximately 1/2 hour, and then kept on ice for 1 hour. The supernatant was removed from the clot and spun at 8,000 x g for 5 min in a minifuge (Biofuge A, Heraeus). The serum was either used immediately in all transfer experiments, or stored in small aliquots at -80°C for use in cell cultures. For 2 experiments in chapter 3, serum samples prepared either from Balb/c or Balb/c SCID mice fed with OVA or PBS under the same conditions, were kindly provided by Elizabeth Furrie.

2.2.4 Fractionation Of Serum Samples

Sera were prepared from OVA-fed or PBS-fed Balb/c mice as described under 2.2.3. For the separation of small molecular weight fragments, sera were
diluted 1/10 and spun through 10kDa size exclusion filters (Millipore, Ultrafree-CL low binding series) at 4°C 5000 x g for approximately 40-60 min. in an Omnifuge 2.0 RS (Heraeus). To wash filters prior to use, they were filled with 2 ml PBS and spun at 4°C 5000 x g for 10 min. This was repeated twice. The filters were subsequently filled with 2 ml of the diluted serum sample. This was 0.2 ml serum plus 1.8 ml PBS for OVA-fed and PBS-fed sera. When sera were spiked with OVA, this was 0.2 ml PBS-fed serum, 1.6 ml PBS, and 0.2 ml of a 200 µg/ml solution (40 µg) of OVA in PBS to yield a concentration of 20 µg/ml OVA in 10% serum. For OVA 323-339, 0.2 ml of PBS-fed serum was mixed with 1.4 ml PBS and 0.4 ml of OVA 323-339 at 1.5 mg/ml (600 µg). Serum samples were spun as mentioned above until approximately 1.5 ml had passed through the filter. Both fractions were further diluted as required for the lymphocyte proliferation assay (figure 3.3), and sterilized by filtering through a 0.2 µm filter (sartorius).

2.2.5. Peptide Synthesis

2.2.5 i Peptide sequences

The sequences of peptides used in studies on EAE in chapters 4-6 were

Ac1-11 (or Ac1-9), Acetyl-ASQKRPSQR(HG)
Ac1-11 (or Ac1-9) [4A], Acetyl-ASQARPSQR(HG)
Ac1-11 (or Ac1-9) [4Y], Acetyl-ASQYRPSQR(HG)

(Ac1-11 respresents the sequence of the N-terminus of rat MBP, Ac1-9 contains the sequence of mouse MBP).

MBP 35-47, TGILDSIGRFFSG
The sequence of OVA 323-339 used for chapter 3 was ISQAVHAAHAEINEAGR.

2.2.5 ii Peptide synthesis

All peptides were synthesized using Fluorenylmethoxycarbonyl-polyamide (Fmoc) chemistry (Atherton & Sheppard, 1985). Peptides of MBP were synthesized by David Wraith on a LKB-Biolynx 4175 synthesizer using NovaSyn KR resin, or provided by Nigel Groome (Oxford). Synthesis on the KR resin yields C-terminal amide peptides which have identical T cell stimulating properties when compared with peptides bearing C-terminal carboxyl groups (David Wraith, unpublished data). Arginine was side-chain protected with the Pentamethylchroman-6-sulfonyl (PMC) group and activated with Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOB) and
diisopropylethylamine. Other L-amino acids were purchased in an ester-activated form, containing appropriate side chain protection groups. Serine was added as the Fmoc-oxo-benzotriazine ester. All other amino acids were pentafluorophenoxy (OPfp) esters. Coupling of each amino acid was followed by counter-ion distribution monitoring (Salisbury et al, 1990) with Novachrome 600 dye on an ULTROSPEC II spectrophotometer (LKB), and allowed to proceed to completion at each step. Peptides were acetylated and subsequently cleaved and deblocked with 95% trifluoroacetic acid (TFA), 5% H₂O for 2 hours. Peptides were precipitated with excess ether and the precipitates washed with ether to remove residual TFA. The dry precipitates were dissolved in 25% glacial acetic acid, lyophilized, dissolved in PBS, filter-sterilized through a 0.2 µm filter (Sartorius), and stored at -80°C.

The peptide OVA 323-339 was synthesized using NovaSyn KA resin and otherwise the same equipment as described above for the synthesis of MBP peptides. Prior to coupling of the first residue (arginine), 0.9 g resin were left to swell for 1 hour in 5 ml Dimethylformamide (DMF). The resin was then carefully mixed with 0.5 ml DMF containing 10.5 mg Dimethylaminopyridine (DMAP, 12 mg DMAP per mg resin). Fmoc-Arg(MTR)-OPFP (0.25mg) was dissolved in 1 ml DMF, mixed with the resin and left at room temperature for 1 hour. The resin was washed twice with approximately 10 ml DMF, which was decanted after each wash. This coupling step was carried out 4x. The resin was subsequently washed 3x with each of DMF, dichloromethane (DCM), propanol, and ether. After washing, the resin was dried for 1/2 hour each on a water and an oil vacuum pump.

To estimate the degree of attachment of arginine to the resin, triplicates of 10 mg dried resin were mixed with 20% piperidine in DMF for 2-3 minutes. After the resin was settled, the absorbance of the supernatant was measured at 290nm. The percentage attachment was read from a standard curve published in the Biolynx users manual. The absorbance (1.84) corresponded to 100% coupling according to the standard.

The resin was then loaded onto the column of the peptide synthesizer. For each coupling step, an appropriate amount of each amino acid (3 x molar excess) was dissolved in 1 ml DMF and added to the loading port together with 0.5 ml hydroxybenzotryazol (HOBT) at 108 mg/ml. Immediately after the amino acid-HOBT solution was applied to the column, 1ml of dye was added, followed by 0.5-1 ml DMF. After each residue, the loading port was washed with methanol and filled with 5 ml DMF. The synthesis was controlled by the 'counter-ion distribution' program.
After completion of the synthesis, the resin was washed 3x with DCM, propanol, and ether, and dried on water and oil vacuum pumps as before. The peptide was then cleaved from the resin by treatment with 95% TFA 5% phenol (1g phenol dissolved in 19 ml TFA) for 24 hours, during which the resin was occasionally mixed. The resin was spun at 1932 x g for 2 min in an Omnifuge 2.0 R5 (Heraeus), and each of two 10 ml samples of the supernatant slowly pipetted to 45 ml ether in plastic tubes (falcon). The precipitate was spun at 1932 x g for 5 min on an Omnifuge 2.0 RS (Heraeus). The pellet was washed 4x in 15 ml ether by vigorous vortexing, and spinning between washing steps at 1932 x g for 5 min as before. After the final wash, the pellets were dried on vacuum pumps as described before. The dry pellets were dissolved in 25% acetic acid (25 ml per pellet), frozen in liquid nitrogen, and subsequently lyophilized for 24 hours. The peptide powder was re-dissolved in water and freeze-dried again. The peptide thus obtained was either stored as a powder at -20°C in a desiccator, or dissolved in PBS, filter-sterilized through a 0.2 µm filter and stored in small aliquots at -80°C.

The HPLC (Waters 600 E) profile revealed a single peak, and the expected amino acid content was confirmed by Len Packman in the department of biochemistry, University of Cambridge (not shown).

2.3 MICE

Female Balb/c mice were purchased from Tucks (UK). PL/J and B10.PL mice were imported from the Jackson laboratories (USA), and bred under specific pathogen-free (SPF) conditions in isolators in the Pathology Department, Cambridge University. (PL/J x B10.PL) F1 mice were also bred in isolators at the same location. All strains were housed in a SPF facility at Central Biomedical Services, New Addenbrooke's site, Cambridge. For all experiments, only female mice were used between 7-14 weeks of age. Groups for each experiment were either of the same age or age-matched.

2.4 METHODS OF ANTIGEN ADMINISTRATION AND ANIMAL PROCEDURES

2.4.1 Feeding

Antigens were dissolved in PBS or 0.15 M NaHCO₃ buffer (pH9). In some experiments (chapter 4) trypsin inhibitor or trypsin/chymotrypsin inhibitor was
included as indicated in the figure legends. When mouse spinal cord homogenate (SCH) was fed, this was suspended in PBS at 10 mg/ml and sonicated for 10 min in a Branson 1200 sonicator. Antigen solutions or SCH suspension were taken up in a 2 ml glass syringe which was then connected to a 1.5 x 22 G dosing cannula with a rounded tip. For feeding, the cannula was carefully inserted approximately 3 cm down into the oesophagus and the required volume of antigen solution (200-500 µl) released from the syringe.

2.4.2 Inhalation

Mice were lightly anaesthetized with ether and laid on their backs. A 25 µl droplet of peptide solution (100 µg) or 25 µl PBS was pipetted on top of their nostrils which was rapidly inhaled as mice regained consciousness.

2.4.3 Immunization

Proteins or peptides were dissolved in PBS. A suspension of H37 RA in PBS was prepared to further enrich complete Freund’s adjuvant (CFA) with mycobacterial antigen. A mixture of antigen solution and H37 RA suspension was then emulsified with an equal volume of CFA by repeated passage through a narrow metal connector between two glass syringes. For priming with SCH, the adjuvant was sonicated for 10 min in a Branson 1200 sonicator prior to passage through the syringes. The quality of the adjuvant was tested by dripping a small amount onto a water surface.

Each mouse was injected with the required dose of antigen in a total volume of 100 µl emulsion subcutaneously at the base of the tail. The doses per mouse were 100 µg OVA, or 50 µg OVA 323-339 in chapter 3, and 50 µg Ac1-9 (Ac1-11), 50 µg MBP 35-47, 1 mg pig MBP, or 1 mg SCH in chapters 4-6. The amounts of H37 RA added to CFA were 100 µg per mouse in all experiments of chapter 3, 400 µg for all studies on EAE in chapters 4-6, and 200 µg for lymphocyte proliferation assays included in chapters 4 and 5.

2.4.4 Induction of EAE

PL/J, B10.PL or (PL/J x B10.PL) F1 mice were primed with encephalitogenic antigens in PBS/CFA subcutaneously at the base of the tail as described under
2.4.3. On the day of immunization and 2 days later, mice were also injected intraperitoneally with 200 ng pertussis toxin in 500 µl PBS. Mice were monitored daily for disease and signs of paralysis analyzed as discussed below.

2.4.5. Transfer Of Spleen Cells

Balb/c mice were fed with OVA or PBS as described under 2.4.1. One week later, spleens were aseptically removed, taken up in either ice cold PBS or IMDM, and single cell suspensions prepared by disrupting spleens with the rubber end of a syringe plunger through a stainless steel wire mesh. Splenocytes in PBS (0.5 ml per spleen) were immediately injected i.p. (see legend to figure 3.10 a). Prior to i.v. injection (figure 3.10 b), spleen cell suspensions in IMDM were spun once for 5 min. at 309 x g 4°C in an Omnifuge 2.0 RS (Heraeus), and resuspended in fresh serum-free IMDM. A small aliquot (30 µl) of cell suspension was mixed with an equal volume of Trypan blue solution and counted for live cells excluding small red blood cells. Spleen cell suspensions (10⁸ live cells in 0.5 ml IMDM) were immediately injected i.v. as described for figure 3.10 b).

2.5 ANÁLISIS DE DATOS DERIVADOS DE ESTUDIOS SOBRE ENCEFALOMIELITIS AUTOINMUNE EXPERIMENTAL

2.5.1 Data Presentation And Analysis

The central questions addressed in chapters 4-6 of this thesis are whether antigen feeding or inhalation have any (protective) effect on EAE. One way of evaluating disease is based on the severity of limb paralysis. This approach was applied to all experiments on EAE presented in this thesis. Paralysis was scored with grades ranging from 0-5 defined as follows:
0 no paralysis
1 flaccid tail
2 partial hind limb paralysis and impaired righting reflex
3 total hind limb paralysis
4 fore and hind limb paralysis
5 death or moribund
Generally, paralysis in EAE progressed from the tail and the hind limbs to the front limbs. Very occasionally, an animal displayed signs of front limb paralysis before the hind limbs were affected. In this case, partial front limb paralysis was also scored as grade 2.

Additionally, weight loss as a result of inflammatory responses and muscle wasting may also be a useful indicator of disease severity. It often precedes the signs of paralysis and can therefore be measured to predict the onset of EAE (that is the onset of paralysis). This aspect is relevant for experiments presented in chapter 6, while the data in chapters 4 and 5 are based on paralysis only.

The course of EAE in each experiment is presented in a plot of mean grade EAE as a function of time. Here, we illustrate the overall severity of disease in a group of mice as a whole on individual days. For this, the cumulative disease score added from the grades of all mice belonging to a group was divided by the number of mice per group. In addition to this approach, there are other parameters that utilize data on the manifestation of EAE in individual mice, such as the incidence of EAE, the day of onset, and the mean maximal severity. These data are presented in a table underneath each plot and subjected to statistical analysis.

The experiments on EAE aimed to investigate whether oral or intranasal administration of antigen had a significant effect on disease. A statistical analysis usually starts with the formulation of the Null Hypothesis Ho, a hypothesis of “no effect”. For this project the Null Hypothesis therefore states that feeding or inhaling antigen has no effect on EAE. An appropriate statistical test is then applied to evaluate whether Ho can be rejected in favour of the alternative hypothesis H1. H1 is the operational statement of our research hypothesis, namely that feeding or inhalation of CNS-derived antigens does have an effect on EAE. Ho is rejected in favour of H1 if a statistical test yields a value whose associated probability p of occurrence under Ho is equal to or less than some small probability. That probability is often referred to as the ‘level of significance’. In many studies including this thesis, a common maximal level of significance is set at p=0.05. In other words, if the probability for Ho being true is equal to or less than 5%, this probability is considered sufficiently small to reject Ho and accept H1 instead.

If our alternative hypothesis stated that feeding or inhalation had a protective effect, then we would already assume that significant differences between controls and treated groups would always reveal less severe disease for treated animals. In other words the hypotheses Ho and H1 already have a defined direction. If we are so sure that the theory underlying H1 permits only one direction, then we will have to consider any opposite effect of treatment, i.e. that
feeding or inhalation exacerbates EAE, as a chance effect which cannot be indicative of a real difference. In this case we would choose to analyze our data with a one-tailed test, since the probability region of the distribution which determines the rejection of Ho (region of rejection) e.g. $p \leq 0.05$ is entirely at one end or tail of this distribution. In contrast, if Ho and H1 are not biased towards a certain direction, then a two-tailed test is appropriate for which the region of rejection is located at both ends of the distribution while maintaining the overall size of this probability region. For instance, if the probability of two selected groups belonging to the same population is 5%, a two-tailed test will yield $p=0.025$ at either end of the distribution (Siegel & Castellan, 1988).

At the beginning of this section, the null hypothesis was stated in general terms without implying a direction, namely that feeding or inhaling CNS-derived antigens has an effect on EAE. Although the term of 'oral tolerance' implies a direction, there are numerous examples in the literature to show that oral administration of an antigen does not always induce tolerance (discussed in chapter 1). Intranasal administration of antigens as a route of tolerance induction is far less well characterized than antigen administration by the intragastric route. Since published data on oral or inhalation tolerance in the H-2u mouse model of EAE were not available, we could not assume that feeding or inhalation would be protective under our experimental conditions. We therefore avoided including a direction in Ho. Consequently, all data were analyzed by two-tailed tests.

2.5.2 Statistical Tests

2.5.2.1 Incidence of disease

Here we asked whether or not a mouse got EAE during the time of the experiment. We only distinguished between sick and healthy, thereby using a nominal or categorical scale. We then examined whether there was a significant difference between any two experimental groups treated in different ways. The Fisher exact probability test is a useful technique for analyzing discrete data when the two independent samples are relatively small, say between seven and 10 subjects per group as in most experiments on EAE. To calculate the p value, the scores were represented by frequencies in a 2x2 contingency table with the column heading indicating the two experimental groups and the row stating the two classifications given by the treatment. The test then determines whether the two groups differ in the proportions with which they fall into the two
classifications. Computation of the test equation provides an exact probability value (Siegel & Castellan, 1988).

2.5.2. ii Median day of onset

The variable "day of onset" may have any value between the induction of EAE on day 0 and the day on which the experiment is terminated. When all animals got EAE, then the differences and distances between any two values were clearly defined and fulfilled the characteristics of an interval scale. This would have permitted the use of parametric statistical tests such as the t-test which are usually more powerful than nonparametric tests. However, animals that remained healthy could not be characterized by a meaningful value within day 0 and the end of the experiment. The day of onset in these cases was described as ' > time of experiment'. This relation no longer fulfilled the requirements for an interval scale. We therefore had to lose some information for statistical analysis and apply an ordinal or ranking scale, instead. The distribution of the values for the day of onset was described by the median, i.e. when the only condition that had to be met was an equal number of scores above and below the median value. To evaluate the differences between days of onset between any two groups, each value within these groups was given a rank, and the significance of the rank sum differences between the groups were evaluated by the Mann-Whitney U-test (Campbell, 1974).

2.5.2. iii Mean maximal EAE

The grades 0-5 constitute an interval scale. This permitted data analysis by a parametric test which focuses on the means of two groups. One standard procedure for comparing the means of two groups is Student's t-test. This test is based on the assumptions that the two groups have 1) a normal distribution and that 2) their variances (square of standard deviations) are the same. The first condition could not be tested since it would have required group sizes of at least one hundred. Fortunately, however, the t-test is said to be relatively "robust" i.e. not much affected by deviations from the normal distribution. From the experience of many EAE experiments we know that the grades of severity often approximate a "normal" distribution with most animals getting disease scores of 2 or 3 and some having mild EAE with grade 1 or more extreme grades in the other direction of 4 or 5. There were, however, examples where some mice got severe EAE of at least grade 3 while the majority of animals remained healthy, i.e. was represented by grade 0. In these cases the standard deviations were greater than the actual means, so that the application of the t-test was
meaningless. In these cases, the values in the tables under "mean maximal EAE" are marked by an asterix to indicate this fact. Obviously, for these examples, the most meaningful parameter to consider is the incidence of disease.

In some experiments the data suggested that the variances between groups were not equal. In these cases the variance ratio test (F-test) was used to evaluate whether the variances between two groups differed significantly at the 5% level ($p \leq 0.025$ for two-tailed probability analysis). When there was a significant difference, the data were analyzed by the Behrens-Fisher test for unequal variances instead of Student's t-test. This is indicated in the figures underneath the tables. Otherwise the given $p$ values were derived from computation of the data according to Student's t-test (Campbell, 1974).

2.6 EVALUATION OF IMMUNE RESPONSES

2.6.1 In Vitro Proliferation Assay For Primed Lymph Node T Cells

This method tests secondary in vitro proliferative responses of lymphocytes obtained from lymph nodes draining the site of subcutaneous antigen/ CFA injection. Although whole lymph node cells were used rather than purified CD4+ T cells, previous reports clearly showed that under the assay conditions described below only primed CD4+ T cells may be activated in vitro (Corradin, Etlinger, & Chiller, 1977; Schrier et al, 1979; reviewed in Taylor, Thomas, & Mills, 1987).

2.6.1 i Preparation of antigens for cell culture

Antigens were dissolved in PBS to 10x the final concentration, sterilized by filtering through a 0.2 µm filter (sartorius), and further diluted in serum free IMDM to twice the final concentration required in the assay. Purified protein derivative (PPD) of mycobacterium tuberculosis was supplied in 5 ml samples which were dialyzed 2x for approximately 12 hours against 500 ml distilled water and also filter-sterilized. Aliquots were kept at -80 °C. In all assays, PPD was included as a positive control, and added to culture wells at a final concentration of 50 µg/ml. Each well contained 100 µl of either the specific test antigen, PPD, or IMDM only as a negative control for proliferation in the absence of exogenous antigen.

2.6.1. ii Preparation of lymphocytes

Mice were primed with antigen in CFA subcutaneously at the base of the tail as described under 2.4.5. Ten days later, the draining lymph nodes (inguinal,
popliteal and paraaortic) were aseptically removed and taken up in ice cold IMDM medium. Single cell suspensions were prepared by disrupting lymph nodes with the rubber end of a syringe plunger through a sterile stainless steel wire mesh. The cells were spun once for 5 min at 309 x g, 4 °C in an Omnifuge 2.0 RS (Heraeus), and resuspended in IMDM supplemented with 1% fresh syngeneic normal mouse serum which was prepared with blood samples from untreated mice as described under 2.2.3. A small aliquot (30 µl) of cell suspension was mixed with an equal volume of Trypan blue solution and counted for live lymphocyte suspensions were then further diluted to a density of 8 x 10^6 live cells/ml and 100 µl of cell suspension, i.e. 8x10^5 cells/well added to 96 well, flat bottomed cell culture plates (Falcon 3072, Becton Dickinson) containing 100 µl of antigen solution. Plates were incubated at 37 °C, 5% CO2 in a LEEC incubator for a total of approximately 90 hours, and pulsed with 0.5 µCurie of tritiated thymidine in 25 µl IMDM per well for the last 20 hours of incubation. The contents of the culture wells was sucked with distilled water onto filter mats on a Skatron Combi cell harvester. The dried filters were soaked with scintillation fluid, sealed into sample bags, and tritium decay counted on a LKB Wallac 1205 betaplate liquid scintillation counter.

For each experiment, samples were plated in triplicates and the arithmetic means of antigen specific proliferative responses illustrated in plots of 3-H thymidine uptake against a titration of antigen. The standard deviations of triplicates, usually between 10-15% of the means, were omitted from the plots for the sake of clarity. The means and standard deviations of control values for medium only and PPD are included in the figure legend or stated beside the plot symbols.

2.6.2 Isotype-Specific Enzyme-Linked Immunosorbent Assay (ELISA)

2.6.2.1 Assay conditions

Balb/c mice were immunized with OVA (see 2.4.3) and serum samples prepared 3 weeks later as described under 2.2.3. The serum was kept at -80°C in small aliquots. For ELISA, sera were diluted in PBS.

Flat-bottomed microtitre plates (Falcon 3912, Becton Dickinson) were coated overnight at 4°C with 50 µl/well of 1 mg/ml ovalbumin in PBS. Plates were washed 2x with PBS and blocked with 200 µl/well of 1% BSA in PBS for 1-2 hours at room temperature (RT). After blocking, plates were washed 6x with PBS containing 0.05% Tween 20 (PBS/Tween). Between the last 3 washes
PBS/Tween was left within the wells for 3-5 minutes. The plates were then incubated for 2-3 hours at RT with 50 µl/well of diluted serum samples and a titration of a standard for subsequent quantitative analysis of serum antibody levels. These standards were either dilutions of a purified anti-OVA IgG (kindly provided by Elizabeth Furrie, London) for the analysis of total IgG, IgG1, and IgG2a, or, for IgM, a standard serum previously shown to contain appropriate amounts of anti-OVA IgM. After washing 6x with PBS/Tween, 50 µl of the detection antibody reagent was added to each well and incubated for 1-2 hours at RT. The isotype-specific antibody reagents had been tested previously against affinity-purified monoclonal antibodies of known isotype (Richard Smith, unpublished data). These reagents were prepared as follows: To detect total IgG within serum samples, a γ-chain specific goat anti-mouse antibody was diluted 1/5000 PBS. This antibody was directly conjugated to horse radish peroxidase (HRP). An HRP-linked goat anti-mouse IgG2a antibody was diluted PBS/1% BSA. The goat anti-IgM reagent was also directly conjugated to HRP and used at 1/1000 dilution in PBS. The rat anti-IgG1 antibody was biotin-linked and used at 1/50 in PBS/Tween containing 1% BSA. After incubation, all plates were washed 6x with PBS/Tween as before. For the biotin-linked anti-IgG1 antibody, 50 µl of HRP-streptavidin diluted 1/1000 in PBS/1% BSA were added to each well and incubated for 30-60 min at RT. Plates were washed 6x with PBS/Tween as before. All plates were subsequently developed with a substrate containing 25 mM citric acid, 25 mM Na2HPO4 in H2O and one 4 mg tablet of 0-phenylenediamine dihydrochloride (ODP) plus 4 µl of 30 % H2O2 per 10 ml substrate solution. The plates were incubated with 100 µl/well of this developing reagent at RT until the colour turned yellow (approximately 5 minutes). The reaction was stopped by adding 50 µl of 1M H2SO4 to each well and the absorbance measured at 492 nm on a Titertek Multiskan Plus (Flow Laboratories). Blank wells contained developing reagent only. The background or nonspecific absorbance for other negative controls (sera from untreated Balb/c mice or wells containing secondary antibodies without serum samples) was negligible.

2.6.2. ii Data analysis

Each plate contained a titration of standard anti-OVA IgG or a standard serum from OVA-primed mice, from which the immunoglobulin concentrations for all test serum samples assayed on the same plate were read. The relative amounts of immunoglobulins in each serum sample were calculated by choosing the serum dilution which yielded an absorbance value within the straight line of
a semi-logarithmic plot of absorbance at 492nm versus standard IgG or standard serum concentration in arbitrary units (for the purified anti-mouse IgG antibody standard, the arbitrary units correspond to µg/ml).

For statistical analysis, the data distribution between the relevant PBS-fed and OVA-fed groups were tested for variance differences (F-test) and analyzed by either the Behrens-Fisher test or Student's t-test as described under 2.5.2 iii.
CHAPTER 3

Oral Tolerance To Ovalbumin

3.1 INTRODUCTION

Oral tolerance to Ovalbumin (OVA) has been extensively studied in mouse models (reviewed in Mowat, 1987), in particular in Balb/c (H-2^d) and (DBA/2xC57BL/6j) F1 (H-2^d xH-2^b) strains. Intragastric administration of OVA suppressed subsequent specific cellular immune responses such as delayed type hypersensitivity (DTH) in vivo (e.g. Miller & Hanson, 1979; Titus & Chiller, 1981; Mowat, 1986; Lamont et al, 1988; Ishii et al, 1993) or secondary in vitro lymphocyte proliferation and IL-2 secretion (Richman et al, 1978; Miller & Hanson, 1979; Melamed & Friedman, 1993 a and 1993 b). The humoral branch of the immune system was also affected as assessed by decreased titres of OVA-specific IgE, IgM, and IgG (Ngan & Kind, 1978; Titus & Chiller, 1981; Mowat et al, 1982; Lamont et al, 1988).

A number of studies showed that specific T suppressor cells accounted for at least part of the mechanism underlying the phenomenon of oral tolerance. The ability to inhibit OVA-specific DTH- and immunoglobulin responses could be transferred to naive recipients with splenocytes from OVA-fed donors (Miller & Hanson, 1979; Lamont et al, 1988). More recently, successful transfer of tolerance to OVA was also achieved with Peyer's patch cells for which the tolerogenic capacity appeared to be abrogated by prior depletion of CD8^T cells (Ishii et al, 1993). Furthermore, administration of drugs thought to specifically affect T suppressor cells such as cyclophosphamide (CY) or 2-deoxyguanosine (2d Guo) prevented the induction of oral tolerance (Mowat et al, 1982; Mowat, 1986; Lamont, 1988). However, tolerance by cell transfer was not readily demonstrated for in vitro proliferation of lymphocytes from OVA-primed recipients. (Challacombe & Tomasi, 1980; Miller & Hanson, 1979). Cell mixing experiments
in vitro with lymphocytes from OVA-fed and control animals also failed to reveal suppression (Richman et al, 1978; Melamed & Friedman, 1993a). Instead, concomitant inhibition of IL-2 production in vitro and diminished expression of IL-2 receptors appeared to be indicative of T cell anergy (Melamed & Friedman, 1993a).

The evidence in favour of a role for CD8+ T suppressor cells suggests that MHC class I-restricted epitopes need to be processed from orally administered OVA. On the other hand, the failure to demonstrate the significance of suppressor mechanisms for T cell nonresponsiveness in vitro implies that suppressor-independent mechanisms also contribute to the phenomenon of oral tolerance. The questions addressed in this chapter concern the relevant fragments or epitopes of the OVA molecule involved in the induction of oral tolerance. An intriguing finding in this respect was the ability to transfer tolerance to naive recipients with serum taken from donors one hour after feeding OVA. Although the nature of this tolerogenic serum factor was not identified, it could be removed by adsorption to a solid-phase anti-ovalbumin matrix (Bruce & Ferguson, 1986a). This observation suggested that the tolerogenic factor shared at least one B cell epitope with the native OVA molecule. On the other hand, analyzing serum samples by ELISA or FPLC gel filtration and anion exchange chromatography, Peng et al showed that the presence of the tolerogen did not correlate with the presence of intestinally absorbed OVA in the serum. The generation of the tolerogen was strictly time dependent and required one hour interval between feeding and serum sampling whereas a form of OVA indistinguishable from the native molecule was detectable as early as 5 min after feeding (Peng, Turner, & Strobel, 1990). Transfer of the OVA-derived tolerogenic serum factor fulfilled some but not all requirements for tolerance induction since serum recipients displayed impaired OVA-specific DTH reactions yet developed normal levels of anti-OVA antibodies (Strobel et al, 1983). In the same report, cyclophosphamide abrogated specific tolerance induction in recipients of serum from OVA-fed donors. These results implied that the tolerogenic serum factor might activate CY-sensitive (T suppressor-) cells. The generation of the serum tolerogen did not require an intact intestinal epithelium. Cyclophosphamide which acts on rapidly dividing cells such as intestinal enterocytes did not abrogate the generation of the tolerogenic factor in serum donors (Strobel et al, 1983). Furthermore, the failure to transfer tolerance with the serum from irradiated OVA-fed mice could be overcome by reconstituting serum donors with normal spleen cells in spite of a severely damaged gut epithelium (Bruce et al, 1987). Another important observation was the failure to generate the tolerogenic factor in SCID mice
suggesting that immunocompetent cells were critical (Furrie, 1992). The evidence for the generation of a yet unidentified tolerogenic factor and the presence of whole OVA in the serum upon OVA feeding suggests that the serum contains both tolerogenic and immunogenic elements. This project investigates both the tolerogenic properties of “OVA-fed” serum in transfer experiments and examines its tolerogenic and antigenic properties for OVA-specific T cell responses in vitro.

The OVA peptide 323-339 represents a major epitope for OVA-specific I-A^d- restricted T cells (Shimonkevitz et al, 1984). It is therefore well suited for studies on oral tolerance to OVA in Balb/c mice (H-2^d) which are among those strains with the most effective suppression of both DTH-and antibody responses (Lamont et al, 1988). This peptide is used to investigate to which extent a MHC class II-restricted epitope can account for phenomena of oral tolerance. If the induction of CD8^+ T cell is essential, then feeding OVA but not OVA 323-339 will induce tolerance. As shown for other models of specific immune suppression, these suppressor cells would probably have to be restimulated at the time of an antigenic challenge (Miller et al, 1993). In this situation, feeding OVA could be expected to downregulate immune responses after immunization with whole OVA but not with OVA 323-339. If suppressor cells are induced and operate through the release of inhibitory lymphokines, they may also downregulate immune responses to a second (not previously fed) antigen in a bystander fashion. For this project, cellular immune responses in Balb/c mice were examined in an in vitro proliferation assay as a suitable method to analyze the relative significance of different antigenic stimuli. Previous work on antibody responses is extended using an isotype-specific ELISA to examine the effect of OVA feeding on differential TH1/TH2 lymphokine regulation and T helper cell populations.

3.2 RESULTS

3.2.1 Antigen-Specificity Of Oral Tolerance To OVA And The Question Of Bystander Regulation

The first experiment tested the antigen-specificity of the effect of orally administered OVA on in vitro proliferative responses of OVA-primed lymphocytes. Among several suitable control antigens, human gamma globulin (HGG) and OVA induced directly comparable levels of proliferation at equimolar concentrations in vitro, whereas responses to human serum albumin and bovine cytochrome c tended to be weaker (not shown). For the experiment
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in figures 3.1 Balb/c mice were fed 20 mg OVA one week prior to priming with either OVA, HGG or both antigens included in the same adjuvant. After 10 days lymphocytes were restimulated in vitro with both antigens in separate cultures. The data presented in figure 3.1 clearly show that there was no nonspecific response of OVA-primed lymphocytes to HGG, and likewise no proliferation of HGG-primed cells with OVA, thus excluding the possibility of cross-reactivity between these two antigens. Proliferative responses to OVA were strongly reduced as a result of prior intragastric administration of OVA. This was true whether OVA-fed mice were primed with OVA alone or with a mixture of OVA and HGG (figure 3.1a). The inhibitory effect of OVA feeding on lymphocyte proliferation was antigen specific since feeding OVA did not affect the response of HGG-primed lymphocytes to HGG (figure 3.1b). The groups that were co-immunized with OVA and HGG were included to address the possibility of bystander regulation. If intragastric administration of OVA induces regulator cells which operate by the release of inhibitory lymphokines, these soluble mediators may impair the functions of nearby T cells in an antigen-independent bystander fashion. Thus, if a second antigen (HGG) is coinjected with antigen that was previously fed (OVA) thereby attracting OVA-and HGG-specific lymphocytes to the same microenvironment, some bystander inhibition of the proliferative response to HGG may be expected. However, the plots in figure 3.1b for PBS-fed or OVA-fed and OVA+HGG-primed lymphocytes are virtually superimposable. The same results were obtained in a second experiment of this kind (not shown). These data demonstrate that feeding a single dose of 20 mg OVA one week before immunization with the same antigen lead to a marked reduction of specific in vitro proliferative responses to OVA. This effect was strictly antigen-specific and also excluded bystander inhibition of the responses to a second antigen (HGG) coinjected with OVA.

3.2.2 Antigenicity Of OVA, OVA323-339 And Serum From OVA-Fed Mice (Ova-Fed Serum).

We then attempted to specify relevant antigenic OVA fragments for OVA-primed lymphocytes. In addition to the previously identified peptide OVA 323-339 we also considered the serum from OVA-fed mice as a potential source of antigenic fragments. It seemed conceivable that the intact OVA present in the serum or the tolerogenic factor itself may evoke proliferation of OVA-primed lymphocytes in vitro. Since the requirements for eliciting secondary immune responses are less stringent than for primary responses (*), an OVA fragment that is tolerogenic for a primary response might nevertheless be able to elicit a

*Fuchs & Katzinger, 1992
secondary response. Thus, the purpose of the experiment presented in table 3.1 was to use the serum from mice that were fed OVA one hour before as a source of OVA-derived antigenic fragments to stimulate in vitro proliferation of OVA-primed lymphocytes. The data shown in table 3.1 clearly reveal the presence of a potent antigenic factor in OVA-fed serum. Since proliferation of lymphocytes from control mice was negligible and serum from PBS-fed mice did not stimulate either control or OVA-primed lymphocytes, this serum factor was clearly derived from OVA, and specifically activated OVA-primed cells.

However, these findings did not yield any information about the nature of the antigenic serum factor (SF). Intestinal tryptic digestion of OVA after feeding might contribute to the generation of (SF). In this case, not only OVA-primed lymphocytes but also lymphocytes from mice immunized with OVA 323-339 should proliferate in response to OVA-fed serum. To address this question, OVA- or OVA 323-339-primed lymphocytes were restimulated in vitro with OVA, OVA 323-339 or dilutions of serum from PBS-fed or OVA-fed Balb/c. As shown in figures 3.2a and b both groups of lymphocytes, whether primed with the whole antigen or the OVA peptide 323-339 proliferated vigorously when incubated with either OVA (figure 3.2a) or OVA 323-339 (figure 3.2b). Although lymphocytes responded more strongly when restimulated in vitro with the homologous antigen, i.e. OVA-primed lymphocytes with OVA and OVA 323-339-primed cells with this peptide, the results clearly demonstrate that a physiologically relevant epitope was generated from OVA that was either identical or cross-reactive with OVA 323-339. In contrast, there was no proliferation above background when OVA 323-339-primed cells were incubated with OVA-fed serum. While OVA-primed cells proliferated strongly when restimulated with serum from OVA-fed mice, the dose response curve to the OVA-fed serum using OVA 323-339-primed cells was identical with the negative controls, i.e. OVA or OVA 323-339-primed cells incubated with PBS-fed serum. These data suggested that OVA 323-339 and SF might represent distinct non-crossreactive epitopes of OVA. Alternatively, a simpler explanation is based on quantitative rather than qualitative differences, namely the much lower sensitivity of peptide-primed versus OVA-primed lymphocytes in response to whole OVA in vitro (figure 3.2a). It is therefore conceivable that OVA-fed serum contained whole OVA at concentrations sufficient to be detected by OVA-primed but not by peptide-primed cells.

To distinguish between these possibilities, OVA-fed serum was spun through a 10 kD molecular size exclusion filter and the < 10 kD and > 10 kD fractions tested against OVA-primed lymphocytes in vitro (figure 3.3). As controls, OVA-primed cells were restimulated with PBS-fed serum spiked with either OVA or
OVA 323-339 (figure 3.3a). To allow for a quantitative estimate, lymphocytes were also tested against OVA or OVA 323-339 (figure 3.3b). Figure 3.3a shows that all > 10 kD fractions elicited significant proliferation. This was expected since the serum samples were not spun to completeness and therefore some < 10 kD fragments such as OVA 323-339 added to PBS-fed serum were retained in the > 10kD fraction. Importantly however, the only < 10kD fraction capable of restimulating OVA-primed cells was derived from OVA 323-339-spiked PBS-fed serum. The data for the < 10 kD fractions of OVA-spiked PBS-fed serum and OVA-fed serum were identical and did not exceed background levels at any of the three titration points. These findings did not prove but were consistent with the possibility of the serum factor being identical with whole OVA. A comparison of the proliferative responses to a titration of OVA in figure 3.3b and to serum fractions in figure 3.3a allowed to estimate the amounts of OVA present in OVA-fed serum: the response to 10⁻³.³ (= 0.0005) parts of fractionated OVA-fed serum (about 14,000 cpm) was equivalent to OVA between 10⁻⁹ M and 10⁻¹⁰ M. This implied that neat serum contained between 2x 10⁻⁶ M and 2x 10⁻⁷ M OVA which is about 8-80µg/ml. This is an overestimate since the volume of the > 10 kD fraction was reduced by spinning to about 1/4 of its original size while OVA was obviously retained. If we correct for this factor then neat OVA-fed serum contained between 2-20µg/ml OVA. To test whether this method of a quantitative estimate was meaningful, the proliferative responses to OVA-spiked PBS-fed serum were also considered. Before spinning, 10% PBS-fed serum was spiked with 20 µg/ml OVA. This was concentrated to about 80 µg/ml after spinning. Hence there was a final concentration of about 4 µg/ml OVA at 10⁻².³ parts fractionated serum. The proliferative response after two ten-fold dilutions (10⁻⁴.³ parts serum fraction) i.e. at 40 ng/ml OVA reached about 17,000 cpm (figure 3.3a). This corresponded to the response to 10⁻⁹ M OVA (figure 3.3b) which is also 40 ng/ml OVA.

In summary, the data presented in this section show that OVA 323-339 includes a physiologically relevant epitope for freshly isolated OVA-primed lymphocytes. Furthermore the serum derived from mice fed with 20 mg OVA one hour before is also strongly antigenic for OVA-primed cells. SF probably exceeds a molecular size of 10 kD and may be identical with whole OVA in the range of 2-20µg/ml in this experiment.
3.2.3 Relative Inhibitory Effects Of OVA Feeding On The Proliferation Of OVA-Primed Lymphocytes In Response To OVA, OVA 323-339 And OVA-Fed Serum

Previous results revealed substantially diminished OVA-specific proliferation as a consequence of prior oral administration of OVA (figure 3.1). We then assessed the relevance of individual OVA-derived epitopes such as OVA 323-339 and the antigenic component contained within OVA-fed serum for this phenomenon.

To compare the inhibitory effects of OVA feeding on the response to OVA 323-339 with respect to whole OVA, mice were fed and subsequently primed with OVA (figure 3.4). When lymph node cells from OVA-fed mice were incubated with either OVA (figure 3.4a) or OVA 323-339 (figure 3.4b) the proliferative response to the peptide was reduced to almost the same degree as to OVA with cpm values reaching about 50% of cultures with cells from control animals. Thus, feeding OVA affected T cells specific for OVA 323-339.

In the following experiment shown in figure 3.5, the effect of OVA feeding on secondary in vitro activation by either OVA (figure 3.5a) or OVA-fed serum (figure 3.5b) was compared. In the previous section we have shown that SF may be identical with intact OVA. We have not, however, excluded the possibility of SF representing an antigenic fragment of OVA with a molecular size bigger than 10 kD but smaller than the whole OVA molecule. In fact, early experiments using 30 kD exclusion filters suggested that SF was smaller than 30 kD (not shown). Coomassie blue or silver-stained protein gels revealed that this filter did indeed faithfully retain serum proteins with a molecular size larger than 30 kD. However, a conclusive analysis was hampered by the finding that theSigma OVA preparation itself contained smaller degradation products which left a smear reaching far below the actual 43 kD band for intact OVA on silver stained protein gels (not shown). Thus, the question as to whether SF were a fragment > 10 kD but smaller than intact OVA and possibly identical with the tolerogenic factor remained open. To further address this question, we explored the interesting finding that tolerance transfer usually done with serum from OVA-fed Balb/c mice proved unsuccessful when the serum for transfer was taken from OVA-fed SCID mice (Furrie, 1992). Therefore, if the antigenic SF were identical with the tolerogenic factor, one would expect OVA-primed lymphocytes to fail to respond to serum taken from OVA-fed SCID mice. Therefore, OVA-primed lymphocytes from Balb/c mice previously fed OVA or PBS were incubated with OVA (figure 3.5a) or assayed against serum from OVA-fed Balb/c, PBS-fed Balb/c and OVA-fed SCID mice (figure 3.5b). The data
reveal two important points: firstly, the proliferative responses to serum from OVA-fed Balb/c and OVA-fed SCID mice were identical with all groups of lymphocytes. Secondly, the degree of inhibition by prior OVA feeding at comparable levels of proliferation (i.e. 5000-25000 cpm) was at least as high with OVA-fed serum (figure 3.5b) as with OVA (figure 3.5a).

To confirm these observations, the assays described for figures 3.4 and 3.5 were repeated within the same experiment to allow for direct comparison of all three relevant antigenic stimuli, i.e. OVA, OVA 323-339 and SF (figure 3.6). Again, OVA-specific lymphocytes strongly proliferated in response to both sera from OVA-fed Balb/c or SCID mice in exactly the same way. This finding also suggested that the factor responsible for tolerance transfer did not have any impact, either stimulatory or inhibitory, on the proliferation of OVA-primed T cells under these conditions. As already shown in the previous experiment, proliferation of OVA-primed cells taken from orally tolerized mice in response to OVA-fed Balb/c or Balb/c SCID serum was substantially reduced compared to controls (figure 3.6c). As expected, OVA-primed cells proliferated strongly when activated with both OVA or OVA 323-339, although in molar terms, whole antigen added to cultures provided a more 100-1000 times more effective antigenic stimulus than the peptide, an observation already described for the experiment presented in figure 3.4. Diminished proliferation with lymphocytes from OVA-fed mice was observed upon restimulation with both OVA (figure 3.6a) and OVA 323-339 (figure 3.6b) confirming previous results.

### 3.2.4 Proliferative Responses Of OVA 323-339-Primed Lymphocytes After Oral Administration Of OVA

Figures 3.4 and 3.6 showed that oral administration of OVA affected T cells specific for OVA 323-339. However, lymphocytes primed with OVA did not proliferate as strongly to OVA 323-339 as lymphocytes primed with just this peptide (figure 3.2). Therefore, to test the relevance of lymphocytes specific for OVA 323-339 in oral tolerance to OVA under more clearly defined conditions, mice were fed OVA and subsequently immunized with OVA 323-339 rather than OVA (figures 3.7). More importantly, with regard to putative MHC class I-restricted T suppressor cells, priming with the I-A\(^d\)-restricted peptide OVA 323-339 will reveal whether or not an antigenic challenge with a single MHC class II-restricted peptide alone is sufficient to demonstrate an inhibitory effect on secondary in vitro lymphocyte responses. The data in figure 3.7 clearly demonstrate that in two experiments, proliferation of OVA 323-339-primed T cells was reduced as a result of OVA feeding. Although the overall degree of
reduction after orally administered OVA was higher in experiment 2 than in experiment 1 (table underneath legend to figure 3.7), proliferative responses appeared to be equally affected whether lymphocytes were restimulated with OVA or OVA 323-339. Together with previous results, these data suggest that the overall pattern and degree of reduction was very similar whether lymphocytes were restimulated in vitro with OVA or OVA 323-339, and equally observed when mice were primed with whole OVA or OVA 323-339 alone.

3.2.5 Relative Inhibitory Effects Of Orally Administered OVA Or OVA 323-339

T cells primed with OVA 323-339 were clearly affected by prior oral administration of OVA. We next examined whether oral administration of OVA 323-339 on its own was sufficient to inhibit lymphocyte proliferation. To address this question, Balb/c mice were fed 20mg of OVA or 1mg of OVA 323-339, an approximately equimolar amount to whole antigen. Proliferation was markedly diminished both in response to OVA (figure 3.8a) and OVA 323-339 (figure 3.8b) when OVA-primed cells were derived from OVA-fed mice. It should be noted, though, that the degree of inhibition might be overestimated in this experiment, since positive control values were less than half of the other two groups. However, oral administration of OVA 323-339 had no effect on secondary in vitro responses of OVA-primed lymphocytes, the plots being virtually identical with buffer-fed controls and directly comparable control values for medium and PPD. It was possible that repeated feeding and an overall higher dose of peptide would be more effective. Therefore, in the next experiment (figure 3.9) 1 mg of OVA 323-339 were fed three times 7, 5, and 3 days before priming with OVA. Another group of mice was fed approximately 20 mg OVA divided into three 7 mg doses administered on the same days as peptide. Under these conditions, proliferation of lymphocytes from OVA-fed mice was substantially reduced whereas the same data plots for lymphocyte proliferation with cells from peptide-fed and buffer-fed control mice were almost superimposable, whether restimulated with OVA or OVA 323-339. Therefore, even though proliferation of OVA 323-339-specific lymphocytes was reduced after oral administration of OVA, this effect could not be achieved by feeding this peptide alone.

3.2.6 Transfer Of Spleen Cells From OVA-Fed Donors And Transfer Of OVA-Fed Serum

We examined whether the in vitro phenomenon of reduced specific lymphocyte proliferation could be induced by adoptive transfer of spleen cells
and with serum from OVA-fed donors. When spleen cells were taken from donors one week after OVA feeding and injected i.p. (figure 3.10a) or i.v. (figure 3.10b) into naive recipients, this had no effect on the ability of recipient lymphocytes to proliferate in response to OVA after priming within 24 or 2 hours upon transfer. The same results were obtained when serum taken from donors that had been fed OVA 1 hour before was transferred i.p. (figure 3.11a) or i.v. (figure 3.11b). While proliferative responses with lymphocytes from mice that had been fed with OVA directly were clearly reduced compared to PBS-fed controls, this effect was not observed upon transfer of OVA-fed serum under the same conditions, i.e. one week prior to an antigenic challenge with OVA (figure 3.11).

3.2.7 Effect Of OVA Feeding On Specific Immunoglobulin Levels

In order to examine possible differential inhibition by orally administered OVA on T helper subsets, immunoglobulin levels were analyzed for IgG isotypes that commonly serve as markers for TH1 and TH2 type responses, IgG2a and IgG1, respectively. For the experiment shown in table 3.2, serum samples were taken from OVA-fed and PBS-fed mice three weeks after priming with OVA. At this late time point, IgM levels were low in all groups. Although IgM concentrations tended to be lower in the OVA-fed groups, the overall levels were too low and the variation within groups too high to show significant differences. In contrast, the data reveal significantly lower levels of all tested gamma immunoglobulins, total IgG, IgG2a, and IgG1 in sera from OVA-fed mice compared to controls. There was no evidence for IgG2a or IgG1 being either more or less affected by oral administration of OVA.

3.3 DISCUSSION

3.3.1 The Nature Of The OVA-Derived Antigenic Serum Factor (SF)

3.3.1 i The data are consistent with SF representing a form of intact OVA

One hour after feeding Balb/c mice with a single dose of 20 mg OVA, their serum was highly antigenic for OVA-primed lymphocytes in vitro (table 3.1). Although the one hour interval between feeding and serum sampling coincided with the time span required to generate the tolerogenic factor in other reports,
this serum tolerogen probably did not contribute to the antigenic properties of the serum. Other studies showed that OVA-fed serum from SCID mice failed to transfer tolerance (Furrie, 1992). However, SCID mouse serum taken one hour after OVA feeding stimulated OVA-primed lymphocytes in exactly the same way as Balb/c OVA-fed serum (figures 3.5 and 3.6).

The OVA peptide 323-339 was originally defined by tryptic cleavage (Shimonkevitz et al, 1984). Since trypsin is released into the small intestine, it seemed possible that this peptide contributed to the antigenic properties of OVA-fed serum. However, the lack of T cell activation in vitro with the fraction passed through a < 10kD size exclusion filter suggested that molecules in OVA-fed serum within this size range and below were not antigenic for OVA-specific T cells (figure 3.3). Rather, the relative proliferative responses observed in this experiment were consistent with SF representing intact OVA in the range of 2-20μg per ml serum. Other assays in this chapter that include overlapping dose responses to OVA and SF allow for additional estimates: In figure 3.5, 25,000 cpm were obtained with $10^{-2.3}$ (= 0.005) parts of OVA-fed serum and with OVA concentrations between $10^{-8}$ and $10^{-7}$ M. This would correspond to 80-800μg OVA per ml neat serum. The same calculation yields 800μg/ml OVA for OVA-fed serum in the experiment of figure 3.6.

3.3.1 ii SF may not be identical with native OVA

Previous attempts to identify the tolerogenic serum factor also considered the possibility that native OVA which had escaped degradation in the gut conferred the tolerogenic properties. In these studies, the amounts of intact OVA in the serum of OVA-fed mice were measured by an radioimmunoassay (Hanson et al, 1977) or ELISA using purified anti-OVA IgG (Bruce & Ferguson, 1986a; Peng, Turner, & Strobel, 1990). The amounts of OVA thus measured ranged between 30-200ng/ml one hour after feeding 20-25mg of OVA to mice. The properties of this form of serum-retrieved OVA detectable by ELISA after FPLC gel filtration and anion exchange chromatography were indistinguishable from the native form of the molecule. However, this (presumably native) form of OVA detectable by ELISA could not explain the tolerogenicity of the serum (Peng, Turner, & Strobel, 1990). Thus, the previously reported concentrations of OVA measured in immunoassays (approximately 30-200 ng) were too small to account for the antigenic properties of SF in our experiments, assuming that SF represented whole OVA at 2-800μg/ml serum.

OVA molecules subjected to gut passage and processing are likely to be denatured or otherwise modified thereby destroying epitopes characteristic of
the native protein to some extent while perhaps exposing others. Therefore, the amount of OVA, even intact OVA may be considerably higher than previous estimates for native OVA based on ELISA.

OVA has served as a model molecule for numerous studies on physicochemical alterations as a function of various solvent conditions and temperature changes. Although OVA molecules could aggregate with varying degrees of polymerisation, the overall spherical conformation of the individual molecule appeared to be rather stable involving only a transition of a very limited portion of a helix to a coil. Nevertheless, certain regions buried within the molecule might be exposed at the surface after denaturation (Koseki et al, 1989). This assumption was subsequently confirmed. The structural differences between native and denatured OVA appeared to be more substantial when subjected to immunological rather than physicochemical methods. The appearance of new antigenic determinants upon denaturation was demonstrated with monoclonal antibodies (MAbs) specific for soluble heat-denatured OVA. Five MAbs that exclusively recognized novel epitopes revealed by denaturation also bound to proteolytic fragments of OVA which - when analyzed by the hydrophathy profile of OVA- were part of both the interior and the exterior of the molecule. (Ikura et al, 1992). Similar differences in antibody recognition of native and denatured OVA have also been reported for urea-denatured OVA (Varshney et al, 1991; Kilshaw et al, 1986). It is therefore possible that the discrepancies between previously reported amounts of native OVA in OVA-fed serum and the estimates for SF as whole OVA discussed in this chapter can be accounted for by some form of denaturation of OVA upon passage through the stomach and small intestine. These structural modifications might have destroyed some B cell epitopes, so that a proportion of whole OVA escaped detection by methods based on antibodies such as in radioimmunoassays and ELISA. In contrast, the biological assay used for this project relied on T cell responses. It is likely that T cell epitopes were processed and presented with comparable efficiency regardless as to whether APC’s encountered OVA in its native or denatured form.

3.3.1 iii Relationship between SF, TF and the phenomenon of oral tolerance to OVA

Previous studies suggested that neither native OVA nor structural modification of the intact molecule as a consequence of passage through the gut could account for the tolerogenic properties of OVA-fed serum in transfer studies. In an attempt to mimic physicochemical alterations as a result of gut processing, 0.1-10 µg of either native, deaggregated or urea-denatured OVA
were injected i.p. Under these conditions native and denatured OVA had no effect on either antibody- or DTH responses whereas parenteral administration of deaggregated OVA suppressed both branches of OVA-specific immune responses (Bruce & Ferguson, 1986b). In another report, intravenous administration of deaggregated OVA over a wide range of doses was never as tolerogenic as pretreatment with OVA via the intragastric route, suggesting that oral tolerance to OVA was not sufficiently explained by the absorption of deaggregated molecules into the blood (Hanson et al, 1977). These findings contrast with tolerance by transfer of OVA-fed serum by which DTH responses were significantly suppressed while antibody titres appear to be unaffected (Strobel et al, 1983).

Although the tolerogenic factor (TF) was generated from orally administered OVA, an intact intestinal epithelium was not a prerequisite for its generation. Severe irradiation damage of the gut epithelium was not responsible for the failure to generate TF, since the loss of tolerogenicity in the serum of irradiated mice could be overcome by reconstituting serum donors with normal spleen cells. Furthermore, TF was generated in cyclophosphamide-treated serum donors although the integrity of the intestinal epithelium was probably disrupted by this drug (Strobel et al, 1983). Thus, the splenocyte reconstitution experiment together with the finding that SCID mice failed to produce TF implied that cellular components of an intact systemic immune system but not the specialized architecture of GALT were required for the generation of TF. The ability to induce tolerance by i.p. injection of TF demonstrated that the mechanism by which TF suppressed DTH responses in recipients probably also operated independently of GALT. However, while pretreatment of OVA-fed serum donors with cyclophosphamide (CY) did not abrogate the tolerogenicity of the serum upon transfer, CY treatment of recipients prior to serum transfer did abolish the ability of OVA-fed serum to inhibit DTH responses to OVA (Strobel et al, 1983). This finding suggested that TF induced regulator cells in recipients which subsequently suppressed DTH reactions.

Two experiments presented in this chapter addressed the inhibitory effect of OVA-fed serum on in vitro T cell proliferation (figure 3.11). Under exactly the same experimental conditions that were reported to inhibit DTH responses in recipients upon i.p. transfer of OVA-fed serum, there was no inhibitory effect on the proliferation of OVA-primed T cells in vitro (figure 3.11a). If TF needed to be a component of the serum, either after OVA feeding or after absorption from the peritoneum into the circulation, then i.v. rather than i.p. injection would perhaps be a more effective route of administration. However, i.v. injection of OVA-fed serum also failed to inhibit T cell proliferation in vitro (figure 3.11b). When
considering a potentially tolerogenic role for SF rather than TF upon transfer, the data show that the amounts of whole OVA (native and/or denatured) present in OVA-fed serum also failed to induce a form of T cell nonresponsiveness that was detectable by in vitro T cell proliferation. In the light of previous studies in other models of peripheral tolerance showing anergy induction by i.v. or i.p. injection of soluble antigens, a tolerogenic effect of SF seemed possible, although in the absence of a more thorough investigation testing various parameters such as dose and timing for serum transfer, we do not know whether it is principally impossible to induce tolerance with SF. However, lymphocytes from other groups of mice that had been fed with OVA directly in the same experiments proliferated less strongly compared to PBS-fed controls. We therefore have to conclude that OVA-fed serum on its own was not sufficient to account for the in vitro phenomenon of reduced T cell proliferation observed after oral administration of OVA.

In summary, previous work and the data presented in this chapter showed that considerable amounts of OVA were present in the blood after intragastric administration of 20-25mg OVA in a form that was highly antigenic for both B cells (presumably native OVA) and T cells (presumably native and denatured OVA). Yet, these forms of OVA did not seem to interfere with the induction of oral tolerance nor could they account for the tolerogenic properties of OVA-fed serum. While in previous reports, the tolerogenic capacity of serum taken one hour after OVA feeding inhibited DTH responses in recipients, we could not demonstrate transfer of tolerance for secondary proliferative responses in vitro under the same conditions.

3.3.2 The Role Of OVA 323-339 In Oral Tolerance To OVA- Anergy Versus Suppression

All studies on lymphocyte proliferation presented in this chapter consistently demonstrated clear reduction of OVA-specific proliferative responses when mice were fed 20 mg OVA one week before priming. This was true for proliferation in response to both whole OVA and OVA 323-339 (figures 3.4 and 3.6). Proliferative responses to OVA 323-339 were also reduced by prior feeding of OVA when T cells were subsequently primed with this peptide alone (figure 3.7). This finding together with a lack of bystander suppression of the response to a second antigen HGG (figure 3.1) argues against a role for those suppressor mechanisms that were shown to mediate oral tolerance in some models of
autoimmune diseases. In the Lewis rat model of EAE, bystander regulation to epitopes of MBP other than the orally administered epitope was mediated by TGF-β released by CD8+ T cells upon feeding and specific stimulation with MBP or the appropriate peptide. However, these CD8+ T cells only suppressed DTH responses when the peptide capable of triggering the release of TGF-β was included as part of a subsequent antigenic challenge (Miller et al, 1993).

Several lines of evidence suggest that the in vitro phenomenon of weaker specific T cell proliferation after OVA feeding reflects a direct suppressor-independent mechanism of oral tolerance consistent with T cell anergy. In addition to the strictly antigen-specific effect of orally administered OVA and the ability to inhibit T cell proliferation after priming with a single I-A^-restricted peptide alone, we could not demonstrate this phenomenon by two procedures that have been shown to induce suppression. Neither the transfer of OVA-fed serum nor transfer of splenocytes either i.p or i.v. from OVA-fed donors affected in vitro proliferation of OVA-primed recipient lymphocytes (figures 3.11 and 3.10). Furthermore, preliminary experiments injecting rat IgG2b anti-CD8 antibodies around the time of feeding under conditions shown to deplete CD8+ cells effectively in mice (reviewed in Cobbold et al, 1992) did not reverse the inhibitory effect of orally administered OVA on in vitro T cell proliferation (not shown). Other studies in which T cell responses were assessed in vitro were also consistent with T cell anergy rather than suppression. Here, the inhibitory effect of orally administered OVA was demonstrated by diminished T cell proliferation, IL-2 production and lack of IL-2 receptor expression (Melamed & Friedman, 1993a). When OVA-primed lymphocytes from OVA-fed and PBS-fed mice were co-cultured, the degree of proliferation of co-cultured cells was in between the level of proliferation measured for each cell population on its own rather than less as would be expected if suppression mediated by lymphocytes from orally tolerized animals had occurred (Richman et al, 1978; Melamed & Friedman, 1993a). In one report, the significance for suppression as a mechanism of oral tolerance to OVA was tested for both in vivo and in vitro immune responses. Here, transfer of tolerance with spleen cells from OVA-fed donors one week after feeding could be demonstrated in recipients for DTH reactions and antibody titres but not for T cell proliferation (Miller & Hanson, 1979). Interestingly, however, when mesenteric lymph node (MLN) cells from donors fed two or four days previously were transferred, there appeared to be some inhibition of vitro proliferation with recipient lymphocytes (Challacombe & Tomasi, 1980). As mentioned before, there is ample evidence for active suppression involved as a relevant mechanism for oral tolerance. Transfer of splenocytes, Peyer's patch (PP) cells or mesenteric lymph node (MLN) cells
from OVA-fed donors inhibited DTH and antibody responses in recipients (Ishii et al, 1993; Richman et al, 1978; Ngan & Kind, 1978; Miller & Hanson, 1979). Suppression by cell transfer was abrogated by 2-deoxyguanosine (Mowat, 1986) or pretreating donor cells with anti-thy 1.2 (Richman et al, 1978) or anti-CD8 but not with anti-CD4 specific monoclonal antibodies (Ishii et al, 1993). Taken together, transfer of active regulatory mechanisms induced in donors by feeding OVA can readily be shown for DTH and antibody responses while mostly failing to inhibit proliferation of the recipients' OVA-specific lymphocytes in vitro.

If T cell anergy alone were responsible for reduced in vitro T cell proliferation, one might expect oral administration of OVA 323-339 on its own to be sufficient for this effect. Surprisingly, this was not the case. Neither a single intragastric dose of 1mg OVA 323-339 nor repeated feeding of 1mg peptide 7, 5, and 3 days before immunization induced a decrease of the proliferative response (figures 3.8 and 3.9). In the same experiments, a single dose of 20 mg OVA or approximately the same amount divided into three doses, i.e. 3x7mg given on the same days profoundly inhibited in vitro proliferation. Two possibilities could account for the failure to inhibit T cell proliferation by feeding peptide alone. Either OVA 323-339 can principally induce anergy when administered via the oral route, but the experimental conditions were not appropriate. Alternatively, other epitopes are required. The amount of 3x1mg peptide given in the second experiment (figure 3.9) not only exceeded equimolar amounts of OVA given to other mice at the same time, it was also well within the range of smaller doses of OVA between 1-5mg shown to inhibit DTH responses in other studies (Lamont, Mowat, & Parrot, 1989). Although protease inhibitors were not included for peptide feeding, the sequence of OVA 323-339 does not contain any specific trypsin or chymotrypsin cleavage sites. It seems therefore unlikely that the oral doses of peptide were to small to be effective. However, current knowledge about the rules that govern anergy are not consistent with the possibility that the doses of fed peptide were too high to induce anergy. Rather, we would have expected higher doses to favour anergy induction. If we therefore propose that anergy by oral administration of OVA 323-339 was either not possible or not sufficient to account for the phenomenon of reduced T cell proliferation observed after feeding whole OVA, we are left with the requirement for the induction of some kind of regulator cell with specificity for an OVA epitopes distinct from OVA 323-339. Previous transfer studies using donor cell population one week after OVA feeding provided some evidence for a role of CD8+ T suppressor cells. However, if results from our preliminary CD8+ depletion experiments hold up in a more thorough analysis, then perhaps CD8+ T cells are involved at a later time point after tolerance induction but not
immediately after feeding. For the depletion of CD8+ cells, anti-CD8 antibodies were injected one day before and one day after priming which had no effect on the inhibition of T cell proliferation by OVA feeding (not shown). This obviously does not exclude the possibility of an early CD4+ regulatory T cell which is perhaps either generated in or migrating to MLN as implied by the observation in one report providing some evidence for reduced in vitro proliferation upon transfer with MLN cells (Challacombe & Tomasi, 1980). Perhaps, even though T cell proliferation was not affected by transfer with spleen cells seven days after donors were fed with OVA, this does not necessarily exclude that CD8+ regulator cells are involved at some stage after direct oral administration of OVA. Further studies are planned to include feeding experiments with the H-2Kb-restricted OVA peptide 229-276 together with OVA 323-339 in C57BL/6 x Balb/c (H-2b x H-2d) mice. Possibly, the class I-restricted peptide which, intriguingly was defined by partial tryptic digestion (Carbone & Bevan, 1989) together with the class II-restricted epitope can substitute for oral administration of the whole OVA molecule. However, if this were true, then the mechanisms of regulation are presumably different from those previously described on the basis of bystander phenomenon and the requirement for reactivation at the time of priming, since the data in this chapter suggest a strictly antigen-specific effect in vitro and sufficient inhibition of proliferation after priming with OVA 323-339 alone. In summary, it is possible that the in vitro phenomena indicative of T cell anergy as shown in this chapter and reported previously such as inhibition of T cell proliferation, IL-2 production and IL-2 receptor expression are the result of some early regulatory mechanism for which anergy is the result rather than the cause, and that several mechanisms, among them anergy, act coordinately in this model of oral tolerance to OVA.

3.3.3 Effect Of Orally Administered OVA On Antibody Responses-Regulation Of TH Subsets

Earlier reports and data presented in this chapter showed that oral tolerance to OVA manifested itself as a reduction or abrogation of both T cell mediated responses and antibody production. Although lower immunoglobulin levels could also be a consequence of direct B cell tolerance, hapten-carrier experiments indicated that a lack of T cell help alone was sufficient to account for impaired immunoglobulin secretion. Mice fed with OVA responded poorly to DNP coupled to OVA but normally to DNP coupled to KLH. On the other hand,
intragastric administration of KLH led to a marked reduction in the response to DNP-KLH (Hanson et al, 1977; Richman et al, 1978; Titus & Chiller, 1981). Furthermore, the inhibitory effect on serum immunoglobulin titres could be overcome by administration of bacterial lipopolysaccharide (LPS) at the same time of feeding or immunization. LPS stimulates B cells directly, thereby probably circumventing the deficiency in T cell-mediated B cell help while leaving other inhibitory effects of antigen feeding on T cell functions unaltered (Titus & Chiller, 1881; Mowat et al, 1986). In addition, intragastric administration of T-independent antigens had no effect on subsequent specific antibody responses (Titus & Chiller, 1981).

One possibility which would also provide a convenient candidate for an early CD4+ regulatory T cell is selective or preferential inhibition of TH1 cells, possibly accompanied by an expansion of TH2 cells which might further downregulate TH1 type responses such as in vivo DTH reactions and IL-2 secretion/proliferation upon secondary stimulation in vitro. However, previous findings and those presented in this chapter are inconsistent with this concept: When levels of IgG2a and IgG1 were analyzed, the overall reduction in total IgG levels as a result of feeding OVA could be attributed to inhibitory effects on both IgG2a and IgG1 isotypes, i.e. indicators of both TH1 and TH2 types responses, respectively (table 3.2). In previous studies, intragastric pretreatment with OVA substantially inhibited IgE (TH2) and IgG (TH1 and TH2) isotypes (Hanson et al, 1977; Ngan & Kind, 1978). Therefore, oral tolerance either affected TH cells at an early stage prior to differentiation into functional subsets or it impaired the B cell helper functions of both TH1 and TH2 cells to a comparable extent.

The IgM levels three weeks after priming were generally very low and the differences between control and OVA-fed groups too small to be statistically significant (table 3.2). Three weeks after priming the IgM levels can be expected to be low unless the main mechanism by which oral tolerance affects the humoral immune responses were inhibition of class switching from IgM to other isotypes. This was obviously not the case. However, when peak levels of IgM one or two weeks after immunization were examined the levels of this early isotype were also reduced as a result of OVA feeding (Mowat et al, 1982). In this study, IgM responses appeared to be more easily inhibited than IgG levels under more stringent experimental conditions such as feeding a small dose of OVA (2mg rather than 20mg) with a longer time interval between feeding and immunization (two weeks instead of one week). In the same study, T cell functions as assessed by DTH responses were only moderately affected under the same feeding and priming regime. Thus, with respect to T cell help, early B cell responses reflected by IgM production might be impaired to some extent but
the insufficient inhibition of T cell functions after low dose feeding and long intervals between feeding and priming might allow the humoral responses to "catch up" and develop normal levels of IgG.

In addition to the apparent discrepancy between in vivo and in vitro phenomena, there also seem to be different effects of OVA feeding on cellular versus humoral in vivo functions as shown for DTH and antibody responses. Transfer of OVA-fed serum inhibited DTH but not antibody responses (Strobel et al, 1983). Administration of 2-deoxyguanosine or cyclophosphamide both abrogated suppression of DTH and antibody responses (Mowat, 1986; Mowat et al, 1982). 2-deoxyguanosine also prevented tolerance transfer by spleen cells (Mowat, 1986). However, cell transfer could not substitute for all effects evoked by direct feeding of OVA: Feeding OVA 7-14 days after priming also suppressed DTH responses but not levels of OVA-specific IgG. Under these conditions 2-deoxy could not abrogate the inhibitory effect of feeding on DTH responses. Furthermore, attempts to transfer 2-deoxy-independent tolerance by spleen cells after priming were unsuccessful (Lamont et al, 1988). These findings suggest that suppressor cells might be involved at an early stage of oral tolerance, presumably by inhibiting the priming of naive OVA-specific T cells. In contrast, when lymphocytes have already encountered antigen in an immunogenic context, mechanisms independent of regulatory cells such as direct induction of anergy in responder cells might operate.

3.4 SUMMARY AND CONCLUSION

Most studies on oral tolerance to OVA address one or more of three T cell functions, namely T helper activity, T cell proliferation and the ability to mediate DTH responses. Many experimental approaches designed to unveil the mechanisms of oral tolerance such as transfer of various cell populations or serum from OVA-fed donors succeeded to reproduce some phenomena of oral tolerance. However, only direct feeding of OVA to mice appeared to consistently affect all three T cell functions. This implies that several mechanisms might operate, not all of which can be induced or revealed with all methods.

In this chapter we showed that 20mg OVA administered orally one week before priming with either OVA or OVA 323-339 alone reduced in vitro proliferative T cell responses to OVA and OVA 323-339 by about 40-80% compared to controls. Some findings presented in this chapter and in previous reports were consistent with T cell anergy as a mechanism to account for impaired T cell proliferation in vitro. These include a strictly antigen-specific
effect of feeding rather than bystander regulation, no apparent requirement for regulatory epitopes at the time of an antigenic challenge, diminished levels of IL-2, and IL-2 receptor expression in vitro, and the failure to demonstrate suppression, either in vivo upon transfer or in vitro in cell mixing experiments. However, other observations cannot readily be explained on the basis of anergy alone. Feeding high doses of OVA 323-339 (3x1mg) had no obvious inhibitory effect on OVA 323-339-specific T cells in vitro. Furthermore, if OVA and OVA fragments absorbed into the circulation induced anergy in the periphery, we would have expected i.p. or i.v. injected OVA-fed serum to inhibit T cell priming in recipients. Even if we exclude a role for TF, native and denatured OVA present in the serum might have induced anergy under these conditions.

It is therefore conceivable that, although in vitro data appear to indicate T cell anergy, this may reflect a consequence rather than the initial mechanism of oral tolerance in this model. Possibly, CD4+ (but presumably not TH2) regulator cells are important at an early stage after feeding, while CD8+ T cells may be more significant later on. This question has to be further addressed in kinetic studies involving selective depletion and transfer of CD4+ and CD8+ T cells at different time points after OVA feeding and oral administration of both MHC class II and class I-restricted OVA peptides under various experimental conditions. Future experiments addressing the requirements for distinct OVA epitopes should compare DTH-, antibody-, and in vitro T cell responses to account for the discrepancies observed between cellular and humoral and in vivo versus in vitro phenomena.
Figure 3.1 Balb/c mice were fed 20 mg OVA in 200μl PBS (closed symbols) or 200 μl PBS alone (open symbols). After 7 days groups of 3 mice were primed with either 100 μg OVA/CFA (circles), human gamma globulin/CFA (HGG, triangles), or a mixture of 100 μg of each OVA and HGG in CFA included in the same adjuvant (squares). Ten days later, lymphocytes from draining lymph nodes were restimulated in vitro with OVA (a) or HGG (b). Proliferation was measured by 3-H thymidine incorporation after 4 days in culture. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Medium and PPD controls are arithmetic means and standard deviations of triplicates in cpm.

Figure 3.1 Specificity Of The Effect Of Orally Administered OVA On Secondary In Vitro Lymphocyte Proliferation
Table 3.1 Testing Serum From OVA-Fed Balb/c Mice As A Source Of OVA-Derived Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>OVA/CFA-primed lymphocytes</th>
<th>PBS/CFA-primed lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>1.8 ± 0.2*</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>PPD</td>
<td>85.3 ± 3.4</td>
<td>121.1 ± 2.9</td>
</tr>
<tr>
<td>40 µg/ml OVA</td>
<td>80.3 ± 11.8</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>4 µg/ml OVA</td>
<td>68.3 ± 7.6</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Serum from OVA-fed Balb/c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>41.0 ± 9.5</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>5%</td>
<td>59.4 ± 5.2</td>
<td>2.6 ± 0.7</td>
</tr>
<tr>
<td>2.5%</td>
<td>69.3 ± 2.6</td>
<td>2.4 ± 0.8</td>
</tr>
<tr>
<td>1.25%</td>
<td>62.6 ± 8.5</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Serum from PBS-fed Balb/c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>1.1 ± 0.5</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>5%</td>
<td>2.6 ± 0.7</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>2.5%</td>
<td>2.4 ± 0.8</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>1.25%</td>
<td>3.0 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
</tbody>
</table>

* mean 3-H thymidine incorporation [cpm/1000] ± SD

Table 3.1 Balb/c mice were primed with 100 µg OVA/CFA or PBS/CFA alone. After 10 days lymphocytes from draining lymph nodes were used as responder cells to test the antigenicity of serum from OVA-fed mice. These sera were prepared from blood samples collected 1 hour after feeding Balb/c with either 20 mg OVA in 200 µl PBS or 200 µl PBS only. Lymphocytes were restimulated with these sera in vitro. OVA, PPD and medium were included as controls. After 4 days of culture, lymphocyte proliferation was measured by 3-H thymidine incorporation.
Figure 3.2 Mice were primed with either 100 µg OVA/CFA (closed symbols) or 50 µg OVA 323-339/CFA (open symbols). Ten days later, lymphocytes from 5-7 mice per group were pooled and restimulated in vitro with OVA (a), OVA 323-339 (b) or dilutions of serum in IMDM. Serum samples were prepared from mice 1 hour after feeding 20 mg OVA (circles, plot c), or PBS (triangles, plot c). The highest concentration of serum was 10^-2.3 parts in IMDM which equals 0.5% serum. Proliferation was measured by 3-H thymidine uptake after 4 days of culture. Symbols represent the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Control values expressed as cpm with medium / PPD were 5730 ± 120 /71,860 ± 6160 for OVA-primed lymphocytes and 6400 ± 0 /72,240 ± 6420 for OVA 323-339-primed cells.
Figure 3.3  Sera were prepared from blood samples obtained 1 hour after feeding 20 mg OVA or PBS, and diluted 1/10 in PBS. In addition, diluted serum samples from PBS-fed mice were spiked with either 20 µg/ml OVA or 300 µg/ml OVA 323-339. All samples were spun through Milipore 10 kD size exclusion filters until about 3/4 of the total volume had passed through the filter. Both fractions i.e. >10kD (a, open symbols) and <10 kD (a, closed symbols) were used to restimulate OVA-primed lymphocytes in vitro as described before. For controls, OVA-primed lymphocytes were restimulated with OVA (b, circles) or OVA 323-339 (b, triangles). Symbols represent the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Medium/PPD controls, mean and SD of triplicates in cpm were 3000 ± 510/33,890 ± 4630.
Figure 3.4 Effect Of Orally Administered OVA On Proliferative Responses of OVA-Primed lymphocytes To OVA And OVA 323-339

Figure 3.4 Four groups of 2-3 mice were fed either 200 µl PBS (open symbols) or 20 mg OVA (closed symbols) 1 week prior to priming with 100 µg OVA/CFA. Ten days later, pooled lymphocytes were restimulated in vitro with either OVA (a) or OVA 323-339 (b). After 4 days proliferation was measured by 3-H thymidine uptake. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Means and standard deviations [cpm] of control cultures are included in the legend.
Figure 3.5 Comparing The Antigenic Properties Of OVA-Fed Serum From Balb/c And SCIDs For Lymphocytes From PBS-Fed And OVA-Fed Balb/c

Figure 3.5 Balb/c mice were fed PBS (open symbols) or 20 mg OVA (closed symbols) 1 week prior to priming with 100 µg OVA/CFA. Ten days later, lymphocytes were restimulated in vitro with OVA (a) or dilutions of serum samples derived from Balb/c or Balb/c SCID mice fed OVA or PBS one hour before blood samples were taken (b). Coded serum samples were kindly provided by Elizabeth Furrie and decoded after the data were available. Symbols of plot b: Serum taken from OVA-fed Balb/c (circles), from PBS control-fed Balb/c (squares), and from OVA-fed Balb/c SCID mice (triangles). Proliferation of lymphocytes was measured by 3-H thymidine uptake. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Control values for medium /PPD in cpm were: 2060 ± 130/46,650 ± 130 for lymphocytes from PBS-fed mice and 1560 ± 60/40,220 ± 4070 for cells from OVA-fed mice.
Figure 3.6 Balb/c mice were fed PBS (open symbols) or 20 mg OVA (closed symbols) and all primed with 100 µg OVA/CFA 7 days later. After 10 days, lymphocytes were restimulated with OVA (a), OVA 323-339 (b), and dilutions of serum (c). For the data shown in plot c), aliquots of serum samples described in the legend of figure 3.5 were used, serum from OVA-fed Balb/c (circles), or from OVA-fed Balb/c SCID mice (triangles). Proliferation was measured as described before. Symbols represent the mean of triplicate samples, standard deviations were less than 20% of the mean. Medium/PPD controls were 3060 ± 250/56,710 ± 4750 for lymphocytes from PBS-fed mice and 2920 ± 280/60,020 ± 6990 for cells from OVA-fed mice.

Figure 3.6 Effect Of Orally Administered OVA On Proliferative Responses of OVA-Primed Lymphocytes To OVA, OVA 323-339, And OVA-Fed Serum

---

**Figure 3.6**

- **a**
  - OVA concentration vs. 3-H thymidine uptake (cpm)
  - PBS-fed mice (open circles) and OVA-fed mice (closed circles)
  - Bars indicate standard deviations

- **b**
  - OVA 323-339 concentration vs. 3-H thymidine uptake (cpm)
  - PBS-fed mice (open circles) and OVA-fed mice (closed circles)
  - Bars indicate standard deviations

- **c**
  - Serum concentration vs. 3-H thymidine uptake (cpm)
  - PBS-fed mice (open circles) and OVA-fed mice (closed circles)
  - Bars indicate standard deviations
Figure 3.7 This figure contains the results of two separate experiments using exactly the same protocol. Plots a) and b) present data of experiment 1, plots c) and d) of experiment 2. Balb/c mice were fed PBS (open circles) or 20 mg OVA (closed circles). Seven days later all were primed with 50 µg OVA 323-339/CFA. After 10 days lymphocyte proliferation was measured in response to OVA 323-339 (a and c) and OVA (b and d) by 3-H thymidine incorporation as described before. Symbols represent the mean of triplicate samples, standard deviations were less than 20% of the mean. Medium/PPD controls [cpm] in experiment 1 were 2980 ± 130/44,050 ± 1460 for the PBS-fed group and 3180 ± 150/47,410 ± 1510 for lymphocytes from OVA-fed mice. The corresponding control data in experiment 2 were 2890 ± 260/59,470 ± 5300 for the PBS-fed group and 1830 ± 180/57,950 ± 7570 for cell samples from OVA-fed mice. For more convenient comparison of the relative effect of OVA feeding on the response to OVA and OVA 323-339, the relative reduction as a result of OVA feeding on proliferation is stated in a table below. % reduction = 100 - \( \frac{\text{cpm OVA-fed}}{\text{cpm PBS-fed}} \).

<table>
<thead>
<tr>
<th>data from plot</th>
<th>% reduction after OVA feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10^{-6} \text{ M})</td>
</tr>
<tr>
<td>exp. 1 a (versus OVA 323-339)</td>
<td>43</td>
</tr>
<tr>
<td>b (versus OVA)</td>
<td>65</td>
</tr>
<tr>
<td>exp. 2 c (versus OVA 323-339)</td>
<td>73</td>
</tr>
<tr>
<td>d (versus OVA)</td>
<td>82</td>
</tr>
</tbody>
</table>

Figure 3.7 Effect Of Orally Administered OVA On The Proliferative Responses Of Lymphocytes Primed With OVA 323-339
Figure 3.8 Comparing The Effect Of Orally Administered OVA Or OVA 323-339 On Proliferative Responses Of OVA-Primed Lymphocytes, I

Within 1 hour, mice were given 2 intragastric doses of 10mg OVA (closed circles) or 0.5mg OVA 323-339 (closed triangles), both in 250 µl 0.15M NaHCO₃ buffer, or 2 x 250µl buffer alone (open circles). After 1 week, all mice were immunized with 100µg OVA/CFA, and 10 days later, lymphocytes pooled from 3 mice per group stimulated in vitro with either OVA (a) or OVA 323-339 (b). Proliferation was measured as described before. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Means and standard deviations of medium and PPD controls [cpm] are indicated as part of the legend.
Figure 3.9 Comparing The Effect Of Orally Administered OVA Or OVA 323-339 On Proliferative Responses Of OVA-Primed Lymphocytes, II

![Graphs showing proliferation responses](image)

**Medium**
- buffer: 2440 ± 260
- OVA: 1960 ± 340
- OVA 323-339: 1980 ± 320

**PPD**
- 45,520 ± 3440
- 42,780 ± 16,380
- 54,880 ± 3440

Figure 3.9 Mice were fed either 1 mg OVA 323-339 (triangles), 7 mg OVA (closed circles) both administered in 200 µl 0.15 M NaHCO₃ buffer, or 200 µl buffer alone (open circles) on days -7, -5, and -3. On day 0, all mice were immunized with 100 µg OVA/CFA, and lymphocyte proliferation assayed in response to OVA (a) or OVA 323-339 (b) 10 days later as described before. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. The equivalent data for medium and PPD controls [cpm] are included in the legend.
Figure 3.10 Effect Of Spleen Cell Transfer From OVA-Fed Donors On Secondary In Vitro Proliferation Of Lymphocytes From OVA-Primed Recipients

Figure 3.10 This figure contains results of two separate experiments. For both experiments, single spleen cell suspensions were prepared from mice that had been fed a single dose of 200µl PBS (open circles) or 20mg OVA (closed circles) 1 week before. In experiment 1 (plot a), spleen cell suspensions pooled from 4 donors were injected immediately i.p. (in 0.5 ml PBS per recipient) into equal numbers of naive Balb/c mice. All recipients were immunized with 100µg OVA/CFA 1 day after cell transfer. For experiment 2 (plot b) spleen cell suspensions pooled from 5 donors were washed twice and counted. Each of 4 naive recipient per group was injected with $10^8$ cells in 0.5 ml IMDM i.v. Two hours later, all were primed with 100µg OVA/CFA. After 10 days, lymphocyte proliferation was assayed as in all other previous experiments. Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Control medium/PPD values for experiment 1 expressed in cpm were 6860 ± 810/ 52,320 ± 5380, and 7080 ± 1470/ 53,360 ± 4260 for lymphocytes from recipients of splenocytes from PBS-fed donors and OVA-fed donors, respectively. The corresponding controls in experiment 2 were 1770 ± 210/ 26,480 ± 2050, and 2440 ± 530/ 38,030 ± 2220.
Figure 3.11 Plots a) and b) show data from two separate experiments. For both assays, Balb/c mice were fed either 20mg OVA or 200µl PBS. 3-4 animals per group were not further treated prior to immunization and represented PBS-fed (open circles) and OVA-fed (closed circles) groups. From the remaining mice, serum samples were prepared 1 hour after feeding. In experiment 1 (plot a), 1ml of serum from either PBS-fed (open triangles) or OVA-fed donors (closed triangles) was injected i.p. into each of 3-4 recipients per group. In experiment 2 (plot b), 0.5 ml serum per recipient was injected i.v. One week later, all groups were immunized with 100µg OVA/CFA and lymphocytes assayed for proliferation as described. Symbols represent the mean of triplicate samples, standard deviations were less than 20% of the mean. Controls in cpm are included in the legend.

Figure 3.11 Comparing The Impact Of Orally Administered OVA And Transfer Of OVA-Fed Serum On The Proliferation Of OVA-Primed Lymphocytes

<table>
<thead>
<tr>
<th>Experiment 1, plot a</th>
<th>Medium</th>
<th>PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-fed</td>
<td>3080±90</td>
<td>35,740±1080</td>
</tr>
<tr>
<td>OVA-fed</td>
<td>1800±580</td>
<td>32,370±530</td>
</tr>
<tr>
<td>PBS-fed, serum transfer</td>
<td>3550±250</td>
<td>19,480±3900</td>
</tr>
<tr>
<td>OVA-fed, serum transfer</td>
<td>2560±240</td>
<td>43,200±4580</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2, plot b</th>
<th>Medium</th>
<th>PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-fed</td>
<td>1820±120</td>
<td>19,480±1380</td>
</tr>
<tr>
<td>OVA-fed</td>
<td>1390±290</td>
<td>14,502±1750</td>
</tr>
<tr>
<td>PBS-fed, serum transfer</td>
<td>1110±50</td>
<td>11,930±1760</td>
</tr>
<tr>
<td>OVA-fed, serum transfer</td>
<td>1680±120</td>
<td>14,090±4550</td>
</tr>
</tbody>
</table>
Table 3.2 Comparing The Effect Of Orally Administered OVA On Serum Levels Of Different Immunoglobulin Isotypes

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunoglobulin concentrations of serum [arbitrary units]</th>
<th>IgM</th>
<th>IgG total (^a)</th>
<th>IgG2a (^b)</th>
<th>IgG1 (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exp. 1 1. PBS-fed, (n=11)</td>
<td>97 ± 34</td>
<td>1250 ± 510</td>
<td>800 ± 510</td>
<td>960 ± 330</td>
<td></td>
</tr>
<tr>
<td>2. OVA-fed, (n=11)</td>
<td>79 ± 14</td>
<td>690 ± 320</td>
<td>360 ± 140</td>
<td>490 ± 350</td>
<td></td>
</tr>
<tr>
<td>exp. 2 3. PBS-fed, (n=11)</td>
<td>166 ± 118</td>
<td>1090 ± 520</td>
<td>370 ± 320</td>
<td>179 ± 133</td>
<td></td>
</tr>
<tr>
<td>4. OVA-fed, (n=10)</td>
<td>105 ± 174</td>
<td>490 ± 390</td>
<td>180 ± 110</td>
<td>78 ± 71</td>
<td></td>
</tr>
</tbody>
</table>

Groups 1 and 2 \(^a_p < 0.01\) (Student’s t-test)  
\(^b_p < 0.02\) (Behrens-Fisher test)  
\(^c_p < 0.01\) (Student’s t-test)

Groups 3 and 4 \(^a_p < 0.01\) (Student’s t-test)  
\(^c_p < 0.05\) (Student’s t-test)

Table 3.2 Groups of 10-11 Balb/c mice were fed 20mg OVA or 200µl PBS. One week later, all were immunized with 100µg OVA/CFA. After 3 weeks, serum samples were prepared from individual mice and assayed for IgM, total IgG, IgG2a, and IgG1 by ELISA. Dilutions of sera and a standard anti-OVA IgG (for IgG, IgG2a, and IgG1) or a standard serum (for IgM) were added to OVA-coated microtitre plates, and serum immunoglobulins detected with isotype-specific secondary antibodies as described in materials & methods. The concentrations of serum antibodies were read at an appropriate serum dilution from the titration plots of standards included on each plate. Serum dilutions for data analysis were 1/100 for IgM, 1/1000 for total IgG and IgG2a, and 1/2000 for IgG1. For statistical analysis, the data distribution between the relevant PBS-fed and OVA-fed groups were tested for variance differences (F-test). Data were analysed using either the Behrens-Fisher test (if variances differed significantly) or Student’s t-test.
CHAPTER 4

Oral Administration Of An Encephalitogenic Peptide And Myelin Protein In A H-2u Mouse Model Of EAE

4.1 INTRODUCTION

Oral tolerance has been studied in various models of autoimmune diseases and viewed as a potential therapeutic approach (reviewed in Thompson & Staines, 1990; Weiner et al, 1994). In numerous reports, a protective effect was critically dependent on the dose of orally administered antigens. In rat models of collagen-induced or adjuvant-induced arthritis, greater suppression of disease was achieved with lower doses of fed collagen (2.5µg or 3µg) than with ten times higher doses (Thompson & Staines, 1985; Zhang et al, 1990). In a mouse model of arthritis feeding 3 mg of collagen had no protective effect while feeding 0.5 mg reduced the clinical severity of arthritis (Nagler-Anderson et al, 1986). In contrast, a recent study on pristane-induced arthritis in mice revealed improved protection with increasing doses of intragastrically administered type II collagen (Thompson et al, 1993). A positive correlation between the amounts of fed target antigen and the degree of protection from disease was also shown for Lewis rat models of EAU and EAE (Nussenblatt et al, 1990; Higgins & Weiner, 1988). Furthermore, different doses and epitopes appeared to selectively trigger distinct mechanisms. In the Lewis rat model of EAE, oral tolerance was achieved with a nonencephalitogenic fragment and - to a lesser extent- also with an encephalitogenic fragment of MBP (Higgins & Weiner, 1988). Subsequently, the suppressive effect of antigen feeding could be attributed to transforming growth factor β (TGF-β) which was released by CD8+ T cells upon antigen-specific stimulation (Miller et al., 1992a). Only non-encephalitogenic epitopes of MBP
triggered the release of TGF-β by CD8+ T cells. Nevertheless, the encephalitogenic epitopes were also capable of inducing oral tolerance by a yet undefined mechanism (Miller et al., 1993). Other investigators failed to demonstrate evidence for suppression in the same model. In one report, reduced in vitro proliferation and IL-2 production of MBP-specific T cells and failure to transfer tolerance were interpreted as evidence for clonal anergy (Whitacre et al., 1991). The discrepancies between these two studies were suspected to arise from different experimental protocols, in particular that the higher amount of fed MBP (4 x 5 mg versus 5 x 1 mg) in the latter study favoured anergy induction over suppression. Recent studies on oral tolerance in EAU demonstrated that the mechanism of oral tolerance was strictly dependent on the dose of fed retinal S antigen (SAg) -derived peptides. High intragastric doses of the pathogenic peptide (5mg/feeding) induced oral tolerance which lacked characteristics of active suppression in as much as it was strictly epitope-specific (no bystander effects) and not transferrable. In contrast, low dose feeding (250µg/feeding) of the same peptide fulfilled these criteria for suppression (Gregerson, Obritsch, & Donoso, 1993). Therefore, while the high dose tolerance could be interpreted on the basis of direct Ts-independent effects on uveitogenic T cells such as anergy or deletion, this explanation did not account for the phenomena of suppression as a result of low dose feeding.

An answer to the central question addressing the nature of the tolerogenic epitopes therefore provides necessary but not always sufficient information about the mechanisms of oral tolerance. If oral tolerance is accomplished with an epitope distinct from the disease-inducing MHC class II-restricted epitope, then the tolerogenic effect on CD4+ T cells cannot solely be accounted for by anergy as a direct effect of peptide-MHC-TCR interactions. In this case, regulatory (suppressor) functions mediated by at least one other type of T cell have to be proposed, with TCR specificity for a tolerogenic, non-antigenic peptide. On the other hand, if the same peptide can be either tolerogenic or antigenic depending on the route of administration, then the concept of anergy may constitute a sufficient mechanistic model of tolerance. However, the significance of CD4+ regulatory T cells also has to be considered.

The H-2u mouse model of EAE is well suited to address the epitope question. Feeding the major encephalitogenic peptide Ac1-9 (or Ac1-11) should reveal whether oral tolerance can be induced with this N-terminal peptide alone. Furthermore, the higher affinity analogues of this peptide with alanine or tyrosine at position four provide useful tools to study the significance of higher avidity MHC-peptide-TCR interactions in oral tolerance. The putative role of distinct regulatory epitopes is tested by oral administration of whole myelin
basic protein purified from pig spinal cord. Porcine MBP and mouse MBP are 90% homologous with the N-termini being identical (Fritz & McFarlin, 1989). Additionally, whole mouse spinal cord homogenate is fed as a rich source of homologous MBP.

4.2 RESULTS

4.2.1 Feeding Ac1-11 And Its Higher Affinity Analogues

In a first series of experiments we investigated whether oral tolerance to EAE was inducible with the encephalitogenic peptide in PL/J mice. A single intragastric dose of 800µg Ac1-11[4K] (which was considered a high dose compared to the amounts of peptides fed in rat models) had no inhibitory effect on disease induced with the same peptide (figure 4.1). However, this negative result was perhaps due to peptide degradation in the stomach and/or small intestine. Furthermore, one study suggested that repeated feeding was more effective than a single dose (Higgins & Weiner, 1988). These aspects were considered in the next experiment in which Ac1-11[4K] was fed three times on days -7, -5, and -2 over a wide range of doses (figure 4.2), with total amounts of 7.5, 75, and 750µg fed peptide. The peptide was mixed with bicarbonate buffer and trypsin inhibitor to avoid proteolysis in the stomach and the intestinal tract. (Ac1-11[4K] has no preferred cleavage sites for chymotrypsin). However, the data of figure 4.2 do not reveal any significant impact of feeding on incidence, onset or severity of disease for any of the groups. The plot shows almost identical disease patterns for the first phase of EAE. However, the possibility that peptide feeding protected against relapses could not be excluded. We subsequently tested either the original peptide Ac1-11[4K] or a higher affinity analogue with alanine at position 4 (figure 4.3). The results demonstrate that neither of the peptides at a total dose of 800µg conferred protection against EAE. Incidence, onset, and severity were virtually identical between groups. In this experiment, there was no tendency of orally administered peptide to protect against relapses. If anything, the group fed with Ac1-11[4K] displayed an aggravated relapse compared with controls.

A possible explanation for the failure to inhibit EAE was that neither the amounts nor the affinities of orally administered peptides sufficed to reach a critical avidity threshold for tolerance induction. In an attempt to force the immune system towards T cell nonresponsiveness, high amounts (3 x 1 mg) of
the highest affinity analogue Ac1-9[4Y] were administered intragastrically in basic buffer and trypsin/chymotrypsin inhibitor. This lead to complete abrogation of in vitro T cell proliferation in response to the encephalitogenic peptide Ac1-9[4K] and conferred full protection from peptide-induced EAE (figure 4.4). In a preliminary experiment to analyze the dose and affinity dependence in more detail, the ability to abrogate in vitro T cell proliferation by feeding 3 x 1 mg of Ac1-9[4Y] was confirmed while the same dose of the 4A analogue and 4K did not appear to have any effect (not shown).

4.2.2 Feeding Whole Myelin Proteins

The peptide feeding experiments revealed that tolerance induced by the intragastric route was difficult to achieve in this mouse model of EAE, and only observed with very high doses of the highest affinity peptide. However, figure 4.2 shows that low doses (previously suggested to induce suppressor mechanisms) also failed to protect animals from disease. It was therefore conceivable that regulatory cells other than CD4+ T cells specific for Ac1-9 were required for suppression in this model. This would entail recognition of distinct epitopes generated from intragastrically administered whole MBP. To test this possibility, mice were fed three times with either 0.1, 0.5, or 1mg (porcine) MBP (figure 4.5). There were no statistically significant differences between any of the groups, although some tendencies were worth noting: the lower doses of MBP appeared to exacerbate disease to some extent in this experiment. The highest dose of 3x1mg MBP possibly influenced the course of EAE. There was a tendency of milder disease during the first phase around day 13 after EAE induction followed by more a pronounced second phase. While the incidence and mean maximal severity were at least as high as in all other groups, the onset of disease seemed to be slightly delayed.

In all experiments shown so far, EAE was induced with the encephalitogenic peptide. However, if regulatory epitopes distinct from Ac1-11 are required to induce oral tolerance, then T cells specific for this epitope probably have to be able to encounter this putative epitope again at the time of priming. Although endogenous MBP may get processed and presented during the course of EAE, oral tolerance may be more readily inducible if mice are primed with whole MBP. The experiment shown in figure 6 addresses this possibility. Mice were fed 3 x 1 mg MBP, and EAE was subsequently induced with either Ac1-11[4K] (figure 4.6a) or MBP (figure 4.6b). When mice were primed with peptide, prior feeding of MBP had no protective effect. Again, as observed before (figure 4.5)
there was a tendency of an aggravated second phase around day 35 in the MBP-fed group. However, in this experiment, there was a moderate increase rather than a decline during the first phase of EAE in the MBP-fed group compared to controls. In contrast, there was an encouraging tendency for fed MBP to protect mice from MBP-induced EAE. The plot indicates a delayed onset, and the data in the table show a tendency for an overall lower incidence, although this was not statistically significant. ($p = 0.075$). However, when the experiment shown in figure 4.6b was repeated in exactly the same way, these impressions were not confirmed (figure 4.7). Rather, intragastric administration of MBP generally appeared to reinforce the establishment of EAE.

While the amino termini containing the major encephalitogenic epitope are identical in mouse and pig MBP, there are some minor sequence differences in other parts of the molecule. It was therefore conceivable that putative regulatory epitopes could either not be efficiently processed and presented by mouse APC or not effectively recognized by T cells. To circumvent this problem, mice were fed with whole mouse spinal cord homogenate (SCH) prior to SCH-induced EAE. However, rather than protecting, intragastric administration of 3 x 2 mg of SCH (containing approximately 3x1mg mouse myelin) lead to a significant aggravation of disease in a first experiment (figure 4.8) while having no effect in a second experiment (figure 4.9).

**4.3 DISCUSSION**

**4.3.1 Intragastric Administration Of The N-Terminal Peptide Of MBP**

The data presented in this chapter demonstrate that the natural encephalitogenic peptide (4K) failed to induce oral tolerance when fed in total doses ranging from 7.5 µg-800µg (figures 4.1 and 4.2). Two intragastric doses of 400µg of the 4A analogue were also ineffective (figure 4.3). In contrast, an attempt to enforce tolerance using high doses (3 x 1 mg) of the peptide analogue with the highest affinity (4Y) revealed complete abrogation of Ac1-9 specific T cell responses as assessed by specific in vitro lymphocyte proliferation and in vivo with respect to EAE (figure 4.4). Although the overall incidence and severity of disease were low in the control group, the complete lack of disease in the 4Y-fed group was nevertheless striking. However, in preliminary experiments to analyze the conditions for oral tolerance with peptide in more detail, feeding the same high amounts (3 x 1mg) of 4K or 4A did not abrogate
specific in vitro T cell proliferation (not shown). We therefore have to conclude that the natural encephalitogenic peptide, and probably also the 4A analogue fail to induce oral tolerance while the high affinity 4Y analogue, the only peptide among the three (4K, 4A, and 4Y) capable of forming stable complexes with the I-A\textsuperscript{a} restriction element (Fairchild et al, 1993) is a powerful tolerogen, at least at very high doses. It will now be necessary to establish the minimal dose of 4Y that can induce tolerance upon feeding.

The mechanism by which the 4Y peptide interfered with T cell priming remains to be established. It is possible that with such high doses of a single epitope, a considerable proportion of 4Y was absorbed into the periphery. Therefore, the mode of tolerance induced in the experiment shown in figure 4.4 may have been determined by the rules for peripheral T cell downregulation in general rather than by GALT. The fact that a high dose of a high affinity peptide was required to demonstrate any tolerogenic effect is consistent with the idea of direct, suppressor-independent T cell anergy. As discussed earlier for the HEL model, an avidity threshold for tolerance induction may not be confined to central thymic tolerance but may also restrict T cell downregulation in the periphery. In the HEL model, a hierarchy of epitope dominance coincided with a hierarchy of tolerogenicity (Cibotti et al, 1992). However, in the H-2\textsuperscript{u} mouse model of EAE, the dominant encephalitogenic epitope of MBP is of such low avidity that a putative avidity threshold might not have been reached by peptide feeding. Perhaps the highly unstable I-A\textsuperscript{u}-Ac1-9 complex formation does not only permit the escape from central tolerance but also prevent the induction of peripheral tolerance. However, the 4Y feeding experiment (figure 4.4) shows that it is not principally impossible to downregulate encephalitogenic Ac1-9-specific T cells by the oral route. Other reports also demonstrate a direct epitope-specific inhibitory effect induced by oral administration of a major CD4\textsuperscript{+} T cell-specific target epitope. A recent study analyzed the role of encephalitogenic and non-encephalitogenic epitopes in oral tolerance (Miller et al, 1993). Three non-encephalitogenic epitopes, 21-40, 51-70, and 101-120 of guinea pig MBP could induce oral tolerance and trigger the release of TGF-\beta. The encephalitogenic peptide 71-90 did not mediate TGF-\beta release, yet it was possible to induce oral tolerance with this peptide, as well. Although the mechanism by which the orally administered encephalitogenic peptide exerted its tolerogenic effect in the Lewis rat model or EAE was not established, it was consistent with clonal anergy. While in this EAE model, different epitopes induced different mechanisms, different doses of the same peptide appeared to trigger distinct mechanisms in the Lewis rat model of EAU. Repeated feedings of low doses (250\mu g) of an S-antigen peptide was protective against EAU induced
with either the same peptide (343-362), whole S-ag or with a distinct pathogenic epitope comprising residues 270-289. Under these conditions, protection could also be adoptively transferred, thereby meeting two general criteria for suppression. In contrast, when high doses of 343-362 of S-ag were fed (5mg/feeding), protection was only achieved against EAU induced with the same peptide and was not transferable, observations consistent with clonal anergy. The cells that mediated suppression under the low dose feeding regime were not identified, but the finding that the same peptide could induce both active suppressor mechanisms and induce CD4+ dependent EAU suggested that the peptide either induced CD4+ Ts cells upon feeding or that it could also bind to class I MHC thereby inducing CD8+ regulator cells (Gregerson et al., 1993). However, in our study, there was no evidence for protection from EAE with any of the doses of intragastrically administered Ac1-11 as shown in figures 4.1-4.3. We therefore have to conclude that low doses of the encephalitogenic peptide failed to induce suppressor mechanisms in this mouse model of EAE.

The terms “high” and “low” are, of course, both vague and relative. At the time when the project on oral tolerance in the PL/J mouse model was started, to our knowledge no reports were available on mouse models of oral tolerance in EAE or any other experimental autoimmune disease. Since the body weight of mice is approximately only 10% of the rat body weight (about 20mg versus 200mg), the effective doses in mice can be expected to differ from those applied in the rat models of oral tolerance. However, the encephalitogenic peptide Ac1-11 was fed three times on days -7, -5, and -2, a time frame known to be effective in the rat model, and over a wide range of doses, namely three feedings of either 2.5µg, 25µg, or 250µg (figure 4.2). This dose range included the amounts that induced suppression in the rat model of EAU (3 x 250 µg) as well as a ten-fold lower dose to account for the differences in body weight between rats and mice. It is, of course, possible that an effective oral dose of Ac1-11 lies within such a narrow range that we missed it in our experiments. Furthermore, effective doses and time frames of feeding are presumably characteristic for each antigen and each model and cannot readily be extrapolated from one system to another.

An alternative explanation for the failure to induce oral tolerance with Ac1-11 or Ac1-9 focuses on the potential role of APC. All reports discussed above use relatively long peptides consisting of about twenty residues. Although Gregerson et al. also report oral tolerance induction by a 9-mer and 13-mer in the EAU model, the clearest suppressive effect appeared to be achieved with the longer peptide 343-362 (Gregerson, Obritsch, & Donoso, 1993). It is not known whether in these experiments, the long peptides bound directly to free class II or class I MHC displayed on the surface of APC or whether they required uptake,
further processing and intracellular association with MHC molecules. In the PL/J model, these requirements apply to Acl-20 since fixed APC fail to stimulate a Acl-11- specific T cell hybridoma (Fairchild et al, 1993). Possibly, the actual process of intracellular antigen handling by APC induces physiological responses in the APC beyond mere antigen proteolysis which in turn influence the outcome of the immune response, for instance by lymphokine release or differential expression of cell surface molecules either on the APC itself and/or on proximate lymphocytes.

\[4.3.2\] Intragastric Administration Of Whole Myelin Protein

The failure to induce oral tolerance with the major encephalitogenic peptide implied that CD4+ T cell mediated regulation could not be induced by the intragastric route in the PL/J mouse model. We therefore tested the significance of distinct regulatory epitopes by feeding whole MBP. As expected, MBP was strongly encephalitogenic in PL/J mice (figures 4.6b and 4.7). Since the major encephalitogenic peptide was obviously processed from MBP in vivo we could test the idea discussed above that this peptide might induce oral tolerance if it were processed from whole MBP.

In the first feeding experiment, EAE was induced with Acl-11 after oral administration of three doses of 1mg MBP. In another study in which 20mg of MBP were fed to Lewis rats, the authors interpret their data revealing inhibition of in vitro T cell proliferation, IL-2 production and lack of tolerance transfer in favour of clonal anergy rather than suppression although suppressor mechanisms were not rigorously excluded (Whitacre et al, 1991). In any case, if higher amounts of fed protein were needed to induce anergy than suppression, the total amount of 3mg MBP fed to mice should have been sufficiently high compared to 20mg MBP fed to rats. The data presented in figures 4.5 and 4.6a demonstrate, however, that oral administration of MBP did not protect against EAE. Furthermore, mice fed MBP displayed some relapse between days 30-40 after disease induction which was not observed in the control groups in either of the two experiments. Figure 4.5 also shows a lack of protection with smaller doses of fed MBP. If anything, 3x100µg and 3x500µg oral doses of MBP caused more severe disease compared to PBS-fed controls.

In the absence of clonal anergy or CD4+ T cell mediated suppression, EAE induction with Acl-11 alone after feeding whole MBP might have hindered the development of alternative suppressor mechanism. Several studies have shown
that “suppressor epitopes” usually need to be included within the antigenic challenge to enable cognate Ts cell mediated downregulation of the responses to other epitopes. For instance, oral administration of the epitope 21-40 of MBP capable of triggering TGF-β release from CD8+ regulator cells only suppressed DTH responses to whole MBP but not to 71-90. Feeding of peptide 21-40, however, inhibited EAE induced by 71-90, postulated to be mediated by 21-40-specific T cells that migrate to the CNS and are subsequently stimulated by peptide 21-40 processed from endogenous MBP (Miller et al, 1993). Nevertheless, we tested the possibility that if the need to process and present regulatory epitopes from endogenous MBP in the CNS were circumvented by challenging with whole MBP rather than only with the encephalitogenic peptide, the induction of suppression might be facilitated. Therefore, in the following experiments shown in figures 4.6b and 4.7, mice were primed with MBP after feeding MBP. The data presented in figure 4.6 suggest that feeding MBP had some protective effect if EAE was subsequently induced with MBP instead of Ac1-11. Although the differences between MBP-fed animals and the control group (groups 4 and 3 in the table, figure 4.6b) fell short of being statistically significant, the apparent delay of disease and the overall lower incidence of EAE in the MBP-fed group were encouraging to further study this phenomenon. However, when this experiment was repeated under exactly the same conditions, there was no indication of oral tolerance. On the contrary, animals fed with MBP appeared to recover less readily after the first phase of EAE and displayed a more chronic disease in contrast to the sharply biphasic course in the control group (figure 4.7).

Although there is 90% homology between pig and mouse MBP, the possibility remained that a relevant regulatory epitope required for the induction of oral tolerance in the mouse was missing or could not be efficiently processed and presented. One report suggested that homologous MBP was a more efficient oral tolerogen than heterologous MBP (Miller et al, 1992b). We therefore decided to feed mouse spinal cord homogenate (SCH) as a rich source of homologous MBP. However, feeding SCH significantly exacerbated disease in one experiment (figure 4.8) and had no obvious effect on the course of EAE in a second experiment (figure 4.9).

4.4 SUMMARY AND CONCLUSION

The data presented in this chapter failed to provide evidence for oral tolerance by the major encephalitogenic peptide Ac1-11 or the higher affinity
analogue 4A in the PL/J mouse model of EAE. Only high doses of the 4Y analogue, capable of forming stable complexes with I-A<sup>u</sup> abrogated EAE and in vitro T cell proliferative responses. The dose dependence for 4Y needs to be established in more detail. To account for the possible need for antigen processing in GALT and the role of distinct regulatory epitopes involved in oral tolerance, subsequent studies included intragastric administration of purified porcine MBP and mouse spinal cord homogenate. However, these feeding regimes did not show any reproducible protective effect, either. While there was one single experiment where feeding MBP tended to delay disease and reduce the incidence of EAE (figure 4.6b), many other experiments did not reveal any effect of feeding (e.g. figures 4.1 and 4.9) or showed a tendency to exacerbate disease at some stage of the experiment (figures 4.2, 4.5, 4.6a, 4.7, and 4.8).

It remains to be established whether feeding even higher doses of Ac1-9[4K] and [4A] or whole myelin proteins can inhibit EAE. It is also possible that antigen was not fed frequently enough or within an appropriate time frame. Furthermore, SCH might contain substances (such as lipids) that interfere with the induction of oral tolerance. Therefore, further studies should employ murine MBP, for which a recombinant form may soon enable the expression of sufficiently high amounts.
Figure 4.1 Oral Administration Of Ac1-11[4K]

Figure 4.1. PL/J mice were fed a single dose of 800µg Ac1-11[4K] in 200µl PBS (closed circles) or PBS alone (open circles). Seven days later, EAE was induced with 50µg Ac1-11[4K]/ CFA.
Figure 4.2 Oral Administration Of Different Doses Of Ac1-11[4K]

![Graph showing oral administration of different doses of Ac1-11[4K]]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3X buffer</td>
<td>6/8</td>
<td>14</td>
<td>2.6 ± 2.1</td>
</tr>
<tr>
<td>3X 2.5 µg</td>
<td>5/7</td>
<td>14</td>
<td>2.0 ± 1.8</td>
</tr>
<tr>
<td>3X 25 µg</td>
<td>5/9</td>
<td>15</td>
<td>2.2 ± 2.2</td>
</tr>
<tr>
<td>3X 250 µg</td>
<td>6/8</td>
<td>13</td>
<td>2.5 ± 1.8</td>
</tr>
</tbody>
</table>

Figure 4.2 PL/J mice were fed the indicated amounts of Ac1-11[4K] in 75 mM NaHCO₃ buffer plus 20 mg/ml trypsin inhibitor or 200 µl buffer plus trypsin inhibitor alone three times on days -7, -5, and -2. On day 0 EAE was induced with 50µg Ac1-11[4K]/ CFA.
Figure 4.3 Comparing The Effect Of Feeding Ac1-11[4K] And Ac1-11[4A]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>8/8</td>
<td>12</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Ac1-11[4K]</td>
<td>6/6</td>
<td>11.5</td>
<td>2.0 ± 0.9</td>
</tr>
<tr>
<td>Ac1-11[4A]</td>
<td>6/6</td>
<td>10</td>
<td>2.0 ± 1.1</td>
</tr>
</tbody>
</table>

Figure 4.3. On days -7 and -5, PL/J mice were fed 400μg of either Ac1-11[4K] (triangles) or Ac1-11[4A] (squares) in 75mM NaHCO₃ buffer including 20mg/ml trypsin inhibitor. Controls (circles) received buffer plus trypsin inhibitor only. EAE was induced with 50μg Ac1-11[4K]/ CFA on day 0.
**Figure 4.4** Effect of high oral doses of Ac1-9[4Y] on in vitro lymphocyte proliferation and EAE

**Figure 4.4** Pl/J mice were fed 1mg Ac1-9[4Y] on days -7, -5, and -3. Peptide was administered in 500µl 150mM NaHCO3 buffer including 2.5 mg/ml trypsin-chymotrypsin inhibitor (closed circles). Controls received 500µl buffer plus protease inhibitors only (open circles). On day 0, the experiment was split to test the effect of peptide feeding on in vitro lymphocyte proliferation (a) and on EAE (b). Figure 4.4a: On day 0, mice were primed with 50µg Ac1-9[4K]/CFA subcutaneously at the base of the tail, as described in material and methods. Ten days later, lymphocytes were restimulated in vitro with Ac1-9[4K], and proliferation measured by 3-H thymidine uptake. Symbols represent the arithmetic mean of triplicates, standard deviations were less than 20% of the mean. Controls for medium/PPD in cpm were 490±40/39,480±660 for lymphocytes from control-fed mice, and 370±70/34,020±1700 with lymphocytes from the peptide-fed group. Figure 4.4b: On day 0, EAE was induced with 50µg Ac1-9[4K]/CFA including 2 injections of PTX as described in material and methods.
Figure 4.5 Feeding Different Doses Of MBP Before Peptide-Induced EAE

![Graph showing the effect of different doses of MBP on the incidence and severity of EAE.](image)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x PBS</td>
<td>5/8</td>
<td>12.5</td>
<td>1.5 ± 1.6</td>
</tr>
<tr>
<td>3x 100µg</td>
<td>7/8</td>
<td>12.0</td>
<td>2.6 ± 1.6</td>
</tr>
<tr>
<td>3x 500µg</td>
<td>7/7</td>
<td>12.5</td>
<td>2.3 ± 1.5</td>
</tr>
<tr>
<td>3x 1 mg</td>
<td>5/5</td>
<td>19.0</td>
<td>2.4 ± 0.9</td>
</tr>
</tbody>
</table>

Figure 4.5. PL/J mice were fed the indicated amounts of porcine MBP or 200µl PBS on days -7, -5, and -2. EAE was induced on day 0 with 50µg Ac1-9[4K] / CFA.
Figure 4.6 Feeding MBP Before Peptide-Induced Or MBP-Induced EAE

Figure 4.6 PL/J mice received 3 x 1mg of MBP in PBS (closed circles) or 200µl PBS alone (open circles) on days -7, -5, and -2. On day 0 EAE was induced with either 50µg Ac1-11[4K]/CFA (figure a) or 1mg porcineMBP/CFA (figure b).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS-fed/ Ac1-11-primed</td>
<td>7/10</td>
<td>2.1 ± 1.3</td>
</tr>
<tr>
<td>2) MBP-fed/ Ac1-11-primed</td>
<td>10/11</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>3) PBS-fed/ MBP-primed</td>
<td>8/10</td>
<td>2.1 ± 1.6</td>
</tr>
<tr>
<td>4) MBP-fed/ MBP-primed</td>
<td>4/10</td>
<td>1.3 ± 2.0 *</td>
</tr>
</tbody>
</table>
Figure 4.7 Feeding MBP Before MBP-Induced EAE

![Graph showing mean grade of EAE over time after MBP treatment.]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>7/9</td>
<td>15</td>
<td>2.1 ± 1.3</td>
</tr>
<tr>
<td>MBP</td>
<td>11/11</td>
<td>15</td>
<td>2.7 ± 1.0</td>
</tr>
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</table>

Figure 4.7 PL/J mice received 3 x 1mg of MBP in PBS (closed circles) or 200µl PBS only (open circles) on days -7, -5, and -2. EAE was induced with 1mg MBP/CFA on day 0.
Figure 4.8 Oral Administration Of Mouse Spinal Cord Homogenate (SCH) Before SCH-Induced EAE, I

![Graph showing mean grade EAE over days after EAE induction for PBS and SCH groups.]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASEa</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>6/10</td>
<td>15</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td>SCH</td>
<td>9/9</td>
<td>14</td>
<td>2.3 ± 0.7</td>
</tr>
</tbody>
</table>

*a p = 0.054

Figure 4.8 PL/J mice were fed 2mg SCH in PBS (closed circles) or 200µl PBS only (open circles) 7, 5, and 2 days prior to EAE induction with 1mg SCH/ CFA.
Figure 4.9 Oral Administration Of Mouse Spinal Cord Homogenate (SCH) Before SCH-Induced EAE, II

![Graph showing mean grade of EAE over time for PBS and SCH groups.]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9/10</td>
<td>14</td>
<td>3.2 ± 1.8</td>
</tr>
<tr>
<td>SCH</td>
<td>8/8</td>
<td>14.5</td>
<td>3.6 ± 1.6</td>
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</table>

Figure 4.9 PL/J mice were fed 2mg SCH in PBS (closed circles) or 200µl PBS only (open circles) 7, 5, and 2 days prior to EAE induction with 1mg SCH/ CFA.
CHAPTER 5
The Effect Of Peptide Inhalation On The Response Of Encephalitogenic T Cells

5.1 INTRODUCTION

In addition to oral tolerance, antigen inhalation has also been revealed as a potentially tolerogenic route. Inhalation of antigen has been demonstrated to specifically downregulate immune responses to soluble proteins such as OVA (reviewed in Holt & McMenamin, 1989), and one report also mentioned that administration of MBP as an aerosol suppressed EAE (Weiner et al., 1991). For our studies, parallel to experiments on oral tolerance, other groups of mice received an intranasal dose of the encephalitogenic peptide Acl-9 (or Acl-11) or its higher affinity analogues 4A and 4Y to examine whether inhalation of a single epitope was sufficient to inhibit EAE. If this were the case, an I-A\textsuperscript{u} restricted direct (suppressor independent) inhibitory effect on CD4\textsuperscript{+} encephalitogenic T cells might operate and be improved with the higher affinity analogues 4A and 4Y. For the first experiments EAE was induced with Ac1-11. To test the inhibitory potential of inhaled peptide further, we subsequently extended this project to disease induced with whole mouse spinal cord homogenate (SCH) which often induces more vigorous disease than peptide alone and also allows for the processing and presentation of additional epitopes. To address the epitope specificity of the immune response mediated by inhaled peptide, we examined the effect of peptide inhalation on secondary in vitro lymphocyte proliferation in response to two well characterized encephalitogenic epitopes, Ac1-9 and 35-47 of MBP.
5.2 RESULTS

5.2.1 Effect Of Peptide Inhalation On EAE Induced With Ac1-11

In a first series of experiments (figures 5.1-5.3) we tested whether the N-terminal peptide of MBP given by inhalation could protect from EAE induced with the same epitope Ac1-11. Mice received a single intranasal dose of 100µg peptide one week prior to EAE induction. Figure 5.1 shows that, even though the incidence of disease was hardly affected, inhalation of the low affinity wild type peptide Ac1-11 significantly reduced disease severity (p<0.01). The effect of the 4A peptide analogue given by inhalation was even more pronounced than protection achieved with the wild type 4K peptide (figure 5.2). Here, inhalation of Ac1-11[4A] almost completely suppressed the pathological signs of EAE. The impact of the 4K peptide on disease was weaker in this experiment and not statistically significant for the data presented in the table. However, there was a tendency for less severe disease in the 4K-pretreated group, as well. This was perhaps most clearly demonstrated in the plot showing a peak in disease severity around day 17 in the control group at which time the mean grade EAE was more than one grade lower in the 4K-pretreated group.

The finding that the 4A analogue was a more powerful tolerogen when administered by inhalation implied a positive correlation between peptide-MHC affinity and tolerogenicity. However, the 4A peptide is not encephalitogenic in the PL/J and (PL/J x B10.PL) F1 strains and only poorly encephalitogenic in B10.PL mice. In contrast, the 4Y analogue and the wild type peptide 4K peptides are equally encephalitogenic in PL/J and (PL/J x B10.PL) F1 mice, and the 4Y peptide appears to be more encephalitogenic in the B10.PL strain (unpublished observations, not shown). Thus, if the tolerogenic effect of inhaled peptide were truly affinity dependent, then we would expect the 4Y analogue to confer the highest degree of inhibition. Figure 5.3 compares the relative protective effects of all three peptides, 4K, 4A, and 4Y. Although the overall severity of EAE in the control group was low when compared to previous experiments, the incidence (9/10) was high. Significant protection as most clearly revealed by decreasing incidence of EAE was achieved with all three peptides and there was a clear positive correlation between peptide-MHC affinity and tolerogenicity.

5.2.2 Effect Of Peptide Inhalation On EAE Induced With Spinal Cord Homogenate

The results described above implied an epitope-specific mechanism of tolerance that was improved by high avidity APC-CD4+ T cell interactions.
However, these findings did not exclude that Acl-11-specific T cells rendered unresponsive to subsequent antigenic challenge with the same epitope were also capable of impairing the encephalitogenic functions of T cells specific for other epitopes such as 35-47 of MBP. Therefore, we next examined whether peptide inhalation could also inhibit EAE induced with a complex mixture of several potential autoantigens as present in whole spinal cord homogenate (SCH). As shown in figure 5.4, the 4A peptide conferred highly significant protection from EAE induced with SCH. This was most clearly revealed by the lower disease incidence (2/10 versus 9/9). Furthermore, the plot indicates that the onset of EAE was delayed by 1-2 weeks in the two animals that got disease in the 4A-pretreated group compared to controls. The tolerogenic effect on SCH-induced EAE was not confined to the higher affinity 4A analogue but was also demonstrated for the original 4K peptide (figure 5.5). Although the incidence of disease was hardly affected, the data presented in the plot and table demonstrate substantially diminished disease severity as a result of 4K inhalation. Furthermore, as already shown for peptide-induced EAE, protection from disease appeared to be improved with the higher affinity 4A analogue with a significant number of mice being completely protected from any clinical signs of EAE (p=0.042).

To investigate this phenomenon further, the tolerogenic properties of all three peptides 4K, 4A, and 4Y on SCH-induced EAE were tested in all three H-2u strains available for our studies, i.e. PL/J, B10.PL, and (PL/J x B10.PL) F1. In the experiment shown in figure 5.6 the inhibitory effect on EAE was confirmed for all three peptides in (PL/J x B10.PL) F1 mice. Perhaps the most significant observation was that inhalation of either of the three peptides completely abrogated relapses in SCH-induced EAE. The plot illustrates that both higher affinity analogues (but not the 4K peptide) also reduced disease severity during the first phase of EAE around days 12-14. Overall, both the 4A and the 4Y peptide significantly reduced the incidence of disease. However, the difference in affinity between 4A and 4Y was not reflected in their tolerogenicity in this experiment.

When this experiment was repeated in the B10.PL strain, unlike in all previous experiments, inhalation of 4K did not appear to affect EAE. The plot of figure 5.7 reveals almost identical disease patterns for control and 4K-pretreated groups. The data in the table show identical incidences of disease and only a marginally lower value for mean maximal grade EAE in the 4K-treated group compared to PBS controls. In contrast, pretreatment with 4A and 4Y by inhalation profoundly ameliorated disease. The data presented in the plot show that the mean grade EAE was at least one grade lower during the peak of disease
on day 13 and at all times thereafter in the groups that had inhaled either of the higher affinity analogues. Although the differences between the 4A and the 4Y treated groups were not substantial, the 4Y peptide appeared to confer slightly better protection than the 4A analogue. Significantly, all mice that did get EAE in the 4Y treated group fully recovered and no relapses occurred. Interestingly, the data reveal a slight relapse in the 4A group between days 32 and 37 which is parallel to the relapse in the control (but not the 4K) group.

In PL/J mice, the patterns of tolerogenicity among the wild type 4K peptide and both higher affinity analogues 4A and 4Y were very similar in peptide-induced (figure 5.3) and SCH-induced EAE (figure 5.8). All three peptides given by inhalation conferred significant protection from EAE with a clear positive correlation between peptide-MHC affinity and tolerogenicity. One critical issue of the experiment shown in figure 5.3 for protection from peptide-induced EAE could have concerned the overall low grade of disease which might have greatly facilitated tolerance induction. This was not a problem in the experiment shown in figure 5.8. Here, not only the incidence of disease but also the overall severity was high, the disease severity in fact being highest among all experiments with SCH-induced EAE. Yet, mice pretreated with 4Y by inhalation were almost completely protected from disease, and substantial inhibition of EAE as well as delay of disease onset was also achieved by inhalation of 4K and 4A.

The unusual pattern of disease in figure 5.8 demands a few additional comments. Typically, EAE in these mouse strains is characterized by a pronounced first phase with a peak around days 12-17. Thereafter, mice may either remain chronically ill at their maximal (rare) or at a lower grade of EAE (common) or recover completely. This phase may be followed by a more or less pronounced relapse which sometimes reaches the peak height of the first phase but is rarely substantially more severe. The experiment of figure 5.8 is a not previously encountered exception from this rule in the sense that a relatively mild first phase of EAE is followed by a rapid exacerbation reaching its peak on day 39, an unusually late time point. Here, disease is far more severe in the second than during the first phase with two grades higher on average. Interestingly, in the 4K treated group, both peaks also occur, albeit with markedly lower average grades and also with delays for both phases, day 19 compared to day 18 and - more clearly- day 45 compared to day 39. None of the 4A or 4Y pretreated animals display clinical signs of EAE until days 39 and 36, respectively. In the 4Y treated group the two mice that got low grade EAE recovered completely while the 4A group displayed a disease pattern parallel to the 4K group but with a lower average grade of EAE. However, in this experiment, data analysis was hampered to a certain extent by the unusually
high number of deaths without prior clinical signs of EAE. The fact that these
deaths occurred at the time of most severe EAE between days 31-39 might have
indicated that deterioration happened too suddenly to be detected within the 24
hours intervals of monitoring. On the other hand, the lack of previous signs of
EAE excludes the classification of these cases as grade 5. Because of this
ambiguity, these cases were stated separately and excluded from statistical
analysis. They do not, however, affect the observations stated above concerning
the relative tolerogenic effects of the three peptide when administered by
inhalation prior to the induction of EAE with SCH.

In summary, a single dose of 100µg Ac1-11(or Ac1-9) and its 4A and 4Y
analogues given by inhalation one week prior to disease induction, protected
mice from EAE induced with either Ac1-11 or SCH. Furthermore, there was a
direct positive correlation between affinity and tolerogenicity in PL/J mice. In
the two EAE experiments done in (PL/J x B10.PL) F1 and B10.PL in which the
inhibitory effects of all three peptides were compared, both higher affinity
analogues inhibited disease more strongly than the 4K peptide, but there was no
clear and reproducible difference between 4A and 4Y.

5.2.3 Effect Of Peptide Inhalation On In Vitro Proliferative
Responses To Encephalitogenic Peptides

The positive correlation between peptide- I-A^a affinity and tolerogenicity
suggested that this form of inhalation tolerance was based on direct interactions
between encephalitogenic T cells and APC without the requirements for
additional suppressor mechanisms. However, the finding that inhalation of Ac1-9
and its higher affinity analogues 4A and 4Y not only interfered with disease
induced by the same epitope but also by whole SCH implied that peptide
inhalation might trigger regulatory mechanisms which could also affect the
functions of other encephalitogenic T cells in a bystander fashion. However,

..
inhibition of the response to 35-47 cannot be explained on the basis of epitope dominance/subdominance.

Previous experiments suggested that the best protection against SCH-induced EAE was achieved by prior inhalation of the 4Y analogue in PL/J mice (figure 5.8). Therefore, if peptide inhalation initiated bystander regulation, it was most likely to be detected in this model. In order to ‘dissect’ the responses to Acl-9 and 35-47 after inhalation of individual peptides and immunizing with either peptide alone of both peptides together, the proliferative responses of primed lymphocytes to these two epitopes were tested in vitro. When mice were immunized with either peptide alone, inhalation of 4Y completely abrogated the proliferative response to Acl-11 (figure 5.9) while restimulation of lymphocytes with 35-47 was not impaired (figure 5.9 b). This experiment also revealed that strong immune responses to MBP 35-47 could be provoked by priming with this peptide (rather than with whole myelin protein). When administered by inhalation, MBP 35-47 inhibited the response to itself but not to Acl-11 (figure 5.10). Here, lymphocytes primed with Acl-11 displayed even higher specific proliferative responses after inhalation of 35-47 compared to PBS controls (figure 5.10 a). However, this was probably due to higher background proliferation in medium alone in this group.

The results presented in figures 5.9 and 5.10 demonstrated strictly epitope-specific inhibition by inhaled peptide in a situation where mice were immunized with individual peptides. Before addressing the question of bystander suppression by inhalation in a co-immunization protocol with a mixture of Acl-11 and 35-47 of MBP, we needed to examine the impact of one co-injected peptide on the specific immune response to the second peptide. It was conceivable that lymphocytes specific for one epitope might benefit from growth factors and lymphokines released upon cognate TCR ligation by T cells specific for the second epitope, i.e. they would receive bystander help. Alternatively, there might be competition between both peptides for presentation by APC. However, since Acl-11 binds to I-A$^{\text{u}}$ and 35-47 is I-E$^{\text{u}}$-restricted, this possibility seemed less likely. Figure 5.11 shows the proliferative responses of lymphocytes from mice primed with a mixture of Acl-11 and 35-47 compared to lymphocytes primed with either peptide alone. The data suggest that the presence of both peptides within the same adjuvant did not affect the priming to individual peptides since the specific responses to Acl-11 (figure 5.11a) and MBP 35-47 (figure 5.11b) were almost identical between groups of lymphocytes. The marginally lower levels of proliferation with Acl-11 of co-primed lymphocytes compared to Acl-11-primed cells, and slightly higher values when assayed against 35-47 can readily be accounted for by moderately lower and higher
medium and PPD controls in the co-immunized group compared with each peptide alone in plots 5.11a and 5.11b, respectively.

Since co-immunization with two peptides per se did not appear to modulate specific T cell responses to individual peptides, we subsequently tested peptide inhalation for bystander inhibition. Mice received an intranasal dose of either Ac1-9[4Y] or 35-47 one week before priming with a mixture of Ac1-9 and 35-47 (figure 5.12). As shown before in figures 5.9 and 5.10, the data reveal complete specific inhibition of the response to Ac1-9 and also lower levels of proliferation with 35-47 after inhalation of this peptide. There also might have been a moderate degree of bystander inhibition of the response to Ac1-9 after inhaling 35-47 and vice versa. However, a conclusive analysis of these data with respect to the significance of the subtle bystander effects was hampered by the fact that the background (proliferation with medium alone) was twice as high in the PBS controls than in both groups that had inhaled peptide. To clarify the issue of bystander effects, the following experiment (figure 5.13) included groups co-immunized with Ac1-9 and 35-47 or primed with either peptide alone. For this experiment, only Ac1-9[4Y] was used for inhalation. Although the control values for medium and PPD varied among groups thus thwarting direct comparisons, the results contain some useful information. As shown before, inhalation of 4Y abrogated the response to Ac1-9 whether Ac1-9 had been injected on its own or in combination with 35-47. Although in the latter case the inhibition was not complete, the background proliferation with medium only was substantially higher in the 4Y-inhaled and co-immunized group compared to lymphocytes from mice that were immunized with Ac1-9 only. Inhalation of 4Y probably did not inhibit 35-47-specific cells nonspecifically when animals had been primed with this peptide alone. Although the level of proliferation against 35-47 was lower in the 4Y-pretreated group, the positive control (PPD) reached only about 50% of the PPD response of the PBS-pretreated group. As to the question of bystander effects, co-immunization with Ac1-9 and 35-47 did not appear to affect the response to either peptide compared to lymphocytes primed with either peptide alone. The response to Ac1-9 was almost identical between individually-and co-immunized groups (figure 5.13 a) with similar medium and PPD controls among these two groups. When restimulated with 35-47, the level of proliferation with lymphocytes from co-immunized mice was slightly lower when compared to lymphocytes from animals primed with 35-47 alone. This difference could be accounted for by lower medium and PPD controls. Taken together, there was clearly no indication of bystander help in this model. Furthermore, inhalation of 4Y did not reduce the in vitro proliferative response to 35-47 when this was co-injected with Ac1-9. In this assay, the response to 35-
47 after 4Y inhalation was even higher compared to PBS-pretreated and co-immunized controls. However, as mentioned before, the higher specific responses in this group were presumably due to higher medium and PPD controls. Therefore, there was probably no net effect of inhaling 4Y on the response of 35-47-specific T cells regardless of whether 35-47 was injected on its own or in combination with Ac1-9.

5.3 DISCUSSION

5.3.1 The Significance Of High Avidity TCR-MHC Class II-Peptide Interactions For Nonresponsiveness Induced By Peptide Inhalation

The data presented in this chapter show that inhalation of the N-terminal encephalitogenic peptide of MBP and its higher affinity analogues markedly inhibited EAE in mice of the H-2^u haplotype. The fact that the encephalitogenic peptide on its own was sufficient to inhibit EAE upon inhalation clearly indicated that suppressor epitopes distinct from the encephalitogenic epitope were not required for this route of immune intervention. In all EAE experiments in PL/J mice there was a clear direct positive correlation between peptide-MHC affinity and the degree of protection from disease. In B10.PL and (PL/J x B10.PL) F1 both higher affinity analogues 4A and 4Y conferred better protection from EAE than the wild type peptide, but the differences between 4A and 4Y were less clear. Since PL/J and B10.PL strains are of identical class II haplotype, genes of the B10.PL background other than those constituting class II MHC may also influence the degree of nonresponsiveness. Furthermore, it is possible that residue four of Ac1-9 has an impact on TCR recognition in addition to its well defined role for MHC-binding. This is supported by the observation that EAE can be induced with the 4A peptide in B10.PL in some (but not all) experiments while this analogue has never been encephalitogenic in PL/J or (PL/J x B10.PL) F1 mice (unpublished data, not shown). Additionally, 4Y induces consistently more severe EAE than 4K in B10.PL whereas in the two other strains 4K and 4Y are equally encephalitogenic even though 4Y but not 4K forms stable complexes with I-A^u (unpublished data, and Fairchild et al, 1993). However, the findings that an encephalitogenic epitope given by inhalation was sufficient to inhibit subsequent immune responses to itself, and that analogues of Ac1-9 with higher affinity for I-A^u were more potent tolerogens than the original low affinity
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peptide imply that high avidity ligation of the trimolecular complex formed by TCR-MHC-peptide is of pivotal importance for T cell inactivation in this model.

Protection from EAE by peptide inhalation was not confined to peptide-induced disease but also to EAE induced with whole spinal cord homogenate (SCH). Two explanations could account for this observation: either there was bystander regulation of the response to epitopes other than the inhaled one or, alternatively, the N-terminal peptide of MBP is such a dominant epitope that immune responses to all other peptides processed and presented from SCH are virtually negligible. Recent evidence argues in favour of the second possibility. In contrast to Ac1-9, MBP 35-47 only induced low incidence and low grade EAE (not shown). Furthermore, when mice were primed with SCH, lymphocytes proliferated strongly when restimulated in vitro with Ac1-9, while the responses to 35-47 were substantially weaker and required much higher peptide concentrations. Another interesting observation in this system was that inhalation of the 4A peptide reduced in vitro proliferation and IFNγ production of SCH-primed lymphocytes to both Ac1-9 and 35-47 while inhalation of 35-47 had no effect on the in vitro response to either peptide (David Wraith, unpublished data). However, data presented in this chapter reveal that inhalation of MBP 35-47 could reduce the proliferative response to this peptide after priming with 35-47 alone (figure 5.10) or co-priming with MBP 35-47 and Ac1-9 (figure 5.12). It is therefore conceivable that the mechanism of nonresponsiveness by peptide inhalation in this model is dependent on strong (dominant) T cell-antigen interactions, not only at the time of inhalation, but also upon the antigenic challenge. This hypothesis could account for the failure to inhibit the subdominant response to 35-47 by inhaling this peptide prior to priming with SCH. In contrast to a challenge with whole antigen, lymphocytes from mice primed with peptides, MBP 35-47 alone or in combination with Ac1-9, proliferated almost equally strongly in response to both peptides (figure 5.11). Thus, when the subdominance of MBP 35-47 was overridden by peptide priming, inhalation of 35-47 reduced the response to itself (figure 5.10) even when the otherwise dominant epitope Ac1-9 was included for immunization (figure 5.12).

Taken together, the positive correlation between peptide-IAb affinity on the one hand, and the apparent need for a dominant T cell response on the other hand suggest that, similar to the HEL model of peripheral tolerance discussed in chapter 1, high avidity interactions of the trimolecular complex TCR-MHC-peptide are required for the induction of nonresponsiveness by peptide inhalation in this model. If this were true, then a gradual increase in avidity by higher amounts of 35-47 should improve the conditions for 35-47-specific T cell
responses to be impaired by the same peptide given by inhalation. For instance, spiking SCH with increasing amounts of 35-47 should then reveal an increasing inhibitory effect of 35-47 upon inhalation.

5.3.2 Mechanism

5.3.2 i Bystander regulation?

A series of experiments presented in this chapter (figures 5.4-5.8) show that peptide inhalation inhibited not only EAE induced with the encephalitogenic peptide but also disease induced with a mixture of several potential antigens such as SCH. This suggests a possibility for bystander effects. Alternatively, if one epitope dominates an immune response to such a large degree as Ac1-9 in this model of EAE, inhibiting the response to this dominant epitope may be sufficient to downregulate autoimmune responses to the whole antigen. However, one finding mentioned in the previous section, namely that Ac1-9[4A] given by inhalation was able to reduce 35-47-specific in vitro responses of SCH-primed lymphocytes (David Wraith, unpublished data) implies a bystander effect. Generally, two explanations could account for this finding: either inhalation of the dominant epitope abrogates bystander help for the development of the subdominant immune response or, alternatively, it actively induces bystander suppression.

The strong immune response to the dominant epitope is probably associated with high concentrations of IFN-γ and IL-2 at some stages of T cell activation. Therefore, whenever T cell responses to subdominant epitopes are initiated within the same micro-environment, they might benefit from the high local concentrations of stimulatory lymphokines triggered by the dominant immune response. In this situation, the response to Ac1-9 would provide bystander help for T cells specific for 35-47. Consequently, inhalation of Ac1-9 might only directly inhibit T cells specific for this same epitope and yet also indirectly impair the response to 35-47 by depriving 35-47-specific T lymphocytes of bystander help. Alternatively, inhalation of the N-terminal peptide of MBP or its higher affinity analogues might create a tolerogenic microenvironment that would mainly affect T cells specific for Ac1-9 but which might also have an impact on adjacent T cells of other epitope specificities. For instance, it is possible that the intranasal route of antigen administration favours TH2 over TH1 type responses. T cells that encounter peptide administered by inhalation might be induced to produce lymphokines such as IL-4 and IL-6 that inhibit
inflammatory TH1-mediated responses central to the manifestation of EAE in vivo or IFN-γ production in vitro. These TH2 type lymphokines might also bias the functions of nearby T cells of other specificities that were attracted to the same site of antigen presentation. Other candidates for inhibitory lymphokines such as TGF-β might also be involved. Although the immunosuppressive role of TGF-β has mainly been discussed in the context of CD8+ Ts cells, TGF-β secretion by CD4+ T cells has not been ruled out. In any case, if peptide inhalation provoked a lymphokine profile that would counteract the manifestation of T dependent inflammatory responses, then inhibition of SCH-induced EAE after inhalation of a single epitope could be explained on the basis of bystander suppression.

In an attempt to analyse how immune responses to Acl-9 and 35-47 influence each other in the absence of a hierarchy of dominance, mice were primed with a mixture of both peptides or either peptide alone. The data in figures 5.11 and 5.13 suggest that co-priming with both peptides did not enhance proliferation in response to individual peptides compared with lymphocyte proliferation after priming with either peptide alone. Therefore, there was no evidence for bystander help under these conditions. Furthermore, inhalation of a single peptide did not appear to induce bystander suppression of the second co-injected peptide. Figures 5.12 and 5.13 demonstrate clear specific inhibition of the proliferative response to the same peptide that had previously been inhaled while there was no significant (suppressive) effect on the response to the second epitope. Taken together, these experiments failed to reveal bystander effects in a situation where a mixture of two peptides was injected. These observations were confirmed in preliminary experiments in another model, for which (BIOZZI x PL/J) F1 mice were primed with a mixture of 56-71 of proteolipid protein (PLP) and Acl-9. Here again, inhalation of each peptide profoundly diminished the in vitro proliferative response to itself but not to the second epitope (not shown). Thus, in a situation in which immune responses to two epitopes are both strong and apparently co-dominant, the phenomenon of inhalation-induced nonresponsiveness appears to be strictly epitope-specific, at least in vitro.

In the case of Acl-9 inhalation prior to priming with SCH, the question as to whether lack of bystander help or the presence of bystander suppression diminished in vitro responses of 35-47-specific lymphocytes remains to be answered. One approach could utilize recombinant MBP for which the nucleotide sequence coding for the N-terminus has been altered by mutagenesis so that Acl-9 can no longer bind to the I-A^u molecule and/or can no longer be recognized by the T cell receptor. If bystander help were important for the activation of 35-47-specific T cells, then the response to 35-47 after priming with
modified MBP should be even weaker compared to priming with wild type MBP bearing the intact dominant N-terminal epitope.

In order to define the mechanism of inhalation-induced immune downregulation, a number of questions will have to be addressed in detail:
1. Where does the peptide locate after inhalation?
2. Which APC are involved in tolerance induction?
3. Can peptide inhalation induce regulator (suppressor) cells?
4. What are the biochemical events in T cells that render them unresponsive to subsequent antigenic challenge?
5. Can inhalation of whole antigen rather than peptide also inhibit T cell activation?
6. Can inhalation modulate an immune response of already primed lymphocytes?

5.3.2 ii. Localization of peptide after inhalation and the role of APC

As discussed in chapter one, studies following the route of inhaled proteins showed that they can line the whole of the respiratory tract (provided the droplet size is appropriate) as well as being detected in the intestinal tract, other organs and the serum. Proteins and dyes that were directly applied to the nasal mucosa rather than in aerosolized form were detected in the lymphatics and the circulation (reviewed in Yoffey & Courtice, 1970; Willoughby & Willoughby, 1977). The procedure adopted for our studies, to directly administer peptide to the nasal mucosa in a volume of 25µ1 probably did not produce droplets of a well defined size upon inhalation. However, this method has been successfully applied for the uptake of influenza virus particles into the lung (Taylor & Askonas, 1986). It seems therefore likely, that intranasally administered peptide is widely distributed within the body, both locally within the respiratory tract and draining lymph nodes, but also in the circulation, thereby gaining access to the spleen. Some of the inhaled peptide probably ends up in the intestinal tract. However, the failure to induce oral tolerance with much higher amounts of Ac1-11[4K and [4A] excludes the possibility of inhaled peptide to induce nonresponsiveness by the intragastric route. The question therefore arises as to which of the several possible routes is important for peptide to exert its tolerogenic effect. Or does sequestration of peptide and encephalitogenic T cells within the lymph nodes draining the respiratory tract provide a trivial explanation for the lack of response after subsequent priming at a different site such as the base of the tail? Further studies using radiolabelled peptide to
evaluate the tissue distribution of inhaled peptide and encephalitogenic T cells in transgenic mice are planned to address this question.

At present, the immunomodulatory properties of Ac1-9 and its higher affinity analogues upon inhalation may be, but need not be explained on the basis of a particular immunosuppressive environment within the respiratory tract. Peptide-induced nonresponsiveness in EAE has been achieved under a variety of conditions. When encephalitogenic MBP peptides were administered in incomplete Freund's adjuvant (IFA) they conferred protection against EAE induced with the same peptide (Smilek et al., 1991; Gaur et al, 1992). In accordance with our observations with inhaled peptide, the higher affinity 4A analogue of Ac1-11 given in IFA inhibited EAE more effectively than the original peptide (Smilek et al, 1991). Likewise, intravenous administration of encephalitogenic peptides coupled to splenocytes induced a form of highly epitope-specific non-responsiveness (Tan, Kennedy, & Miller, 1992). Furthermore, the specific inhibition of EAE and in vitro lymphocyte proliferation achieved by intraperitoneal injection of Ac1-9 and the 4A and 4Y analogues closely resembles the results obtained from peptide inhalation experiments (George Liu, submitted for publication). Taken together, these findings can all be accounted for if the induced nonresponsiveness were a direct consequence of interactions between T cells and peptide-MHC complexes under conditions that fail to provide certain additional secondary signals required to elicit an immune response (Schwartz, 1992).

Recent evidence suggests that immune responses to soluble proteins are also downregulated locally in the lung and upper airways. Dendritic cells (DC) from airway epithelium and lung parenchyma were found to be functionally impaired in their ability to activate T cells. Alveolar macrophages appeared to mediate the inhibitory effect on adjacent DC both in vitro and in vivo (Holt et al, 1993). It is therefore conceivable that, in our model, inhaled peptide was presented by functionally impaired DC and/or alveolar macrophages, a mode of antigen presentation which might render potentially encephalitogenic T cells refractory to subsequent antigenic stimuli.

5.3.2 iii Inhalation of whole antigen and the implication for suppression

The results from our studies in the H-2u mouse model suggest that high avidity TCR-MHC-peptide interactions are favourable to induce nonresponsiveness by peptide inhalation. Since inhalation of a single MHC class II-restricted peptide is sufficient, it is unlikely that CD8+ T suppressor cells are
involved in this model. This contrasts with a rat model of tolerance induction with aerosolized whole OVA. Here, tolerance could be adoptively transferred with class I-restricted CD8+ T cells. The induction of these suppressor cells, however, was preceded by a first phase of a class II-dependent and CD4+ T cell mediated Th2 type response (McMenamin & Holt, 1993). Although the models of inhalation tolerance in this report and our own studies are quite different, it is possible that inhalation of whole myelin antigens might enable additional, MHC class I-restricted and CD8+ T cell-mediated regulation beyond the inhibitory effects achieved with the dominant epitope of MBP alone.

Recently, inhalation tolerance has also been studied in the Lewis rat model of EAU (Dick et al, 1993). The observations reported by Dick et al. are interesting to compare with our experiments in as much as they also adopted direct intranasal inoculation of antigen into the nostrils under light ether anaesthesia rather than continuous exposure to an aerosol. Under these conditions, inhalation of whole retinal antigen (RE) protected against EAU induced with either RE or S-antigen (S-ag). However, their data did not reveal bystander effects since inhalation of S-ag only protected against EAU induced with S-ag but not with RE. This does not necessarily suggest distinct mechanisms of inhalation tolerance in the Lewis rat model of EAU and the H-2u mouse model of EAE. Rather, dominance and subdominance of antigenic epitopes might be less pronounced in EAU than in our model of EAE. Consequently, as suggested by our in vitro proliferation data after priming with peptides (figures 5.11 and 5.13) bystander effects may not significantly influence an immune response where epitopes are co-dominant.

It is therefore desirable to extend our studies on inhalation tolerance to whole myelin antigens. These might initiate further - possibly class I-restricted-regulatory pathways to induce more potent tolerance than achievable by peptide alone under some circumstances, especially with co-dominant responses to several autoantigenic epitopes. Furthermore, inhalation of whole antigen will be the only option to modulate immune responses for which the (dominant) class II-restricted epitopes have not been identified.

5.3.2. iv Effect on T cells

The previous sections emphasized the central role of the trimolecular complex of MHC-peptide-TCR in the induction of inhalation tolerance in our model. Further studies will now have to address the molecular basis underlying this phenomenon of nonresponsiveness. Although this remains largely speculative at present, other models of peripheral tolerance have provided some
insight into peripheral T cell regulation which might also contribute to our understanding of immune manipulation by peptide inhalation.

The inhibition of EAE in vivo and proliferation and IFNγ production in vitro after peptide inhalation reflect the downregulation of TH1 type immune responses. As discussed in detail in chapter one, several previously reported findings could account for this phenomenon. Antigen presentation in the absence of co-stimulatory signals may selectively affect TH1-mediated responses while TH2 cell functions may be independent of such co-stimulation. In the absence of further regulatory mechanisms, antigen presentation that favours T cell anergy over activation can be expected to result in a strictly epitope-specific mode of peripheral tolerance. The data presented in figures 5.12 and 5.13 support this mechanism of nonresponsiveness. Here, inhalation of one peptide only reduced the proliferative response to the same peptide even when another peptide was included in the same adjuvant for priming.

The interlinked biochemical pathways that convert the message of TCR ligation into intracellular signalling cascades have been outlined in chapter one. Considering the delicate balance of these finely tuned inter-related signalling pathways, it is conceivable that perturbation of this intracellular communication network alone may be sufficient to account for differential outcomes of TCR ligation. For instance, moderate degrees of TCR ligation may allow for activation signals to prevail and enable the expression of IL2 concentrations which are appropriate for T cell activation and proliferation. In this situation, negative regulatory mechanisms will ensure that IL2 does not reach toxic levels which would inhibit further T cell function. In contrast, persistent antigenic stimulation may overcome these control functions. Furthermore, the modulatory potential of second messenger pathways involving the metabolism of IP3 and cAMP and the replenishment of Ca2+ stores may be exhausted under persistent and strong TCR-mediated stimulation (Gajewsky et al, 1994). These events may form the molecular basis of some phenomena of specific T cell nonresponsiveness, following super-optimal avidity of the MHC-peptide-TCR complex achieved by high doses of antigen and/or with high affinity ligands (as suggested by data presented in this chapter). The time factor may also constitute another significant parameter: Different time intervals between a first antigenic encounter and subsequent TCR re-engagement may lead to either further activation or nonresponsiveness depending on the physiological “history” of the T cell.

Obviously, for Acl-9 and its higher affinity analogues administered by inhalation, the amount of 100µg given one week prior to an antigenic challenge was appropriate to induce nonresponsiveness. Furthermore, if the hypothesis
discussed earlier were true, namely that dominant or at least sufficiently strong T cell responses at the time of priming are required for the manifestation of inhalation tolerance, this would also emphasize the significance of efficient TCR re-engagement. However, since these ideas focus on the MHC-peptide-TCR complex linked to intracellular signalling events as one major factor of inhalation-induced nonresponsiveness, we may expect other ligands that form MHC-peptide-TCR complexes of different avidities to require different doses and/or timing for the induction of nonresponsiveness by inhalation. Preliminary experiments in other models suggest that the nature of this trimolecular complex might indeed be more important than putative universal immunosuppressive conditions for antigens applied via the mucosa of the respiratory tract. For instance, the same conditions of peptide inhalation that induce nonresponsiveness in the H-2^k mouse model of EAE did not modify the in vitro proliferative responses to OVA 323-339 or OVA after inhalation of OVA 323-339 (not shown). Preliminary studies in an H-2^d mouse model of EAE suggest that tolerogenic doses of inhaled peptides have to be defined for each model and each peptide (David Wraith, unpublished observations).

Thus, peptide inhalation does not appear to lead to tolerance induction by default, and further insight into the rules that govern the fate of specific T lymphocytes when peptide antigens are administered via the mucosa of the respiratory tract will be crucial. Further studies are now planned to investigate the underlying biochemical events such as Ca^{2+} fluxes, and IP3 and cAMP levels.

The bystander effect on the response to 35-47 after priming with SCH and inhalation of Ac1-9 can be sufficiently explained by the abrogation of bystander help otherwise provided by the response to the dominant epitope. However, bystander suppression cannot be excluded. Any binding of Ac1-9 that is detectable by FACS analysis is inhabitable by anti I-Au antibodies (David Wraith, unpublished observation). It is therefore unlikely that this peptide can induce MHC class I-restricted CD8^+ T suppressor cells. However, CD4^+ T cells may also actively downregulate the function of other T cells. In particular, TH2 type cells can be regarded as suppressor cells for TH1-mediated responses. Their action based on antagonistic lymphokines might also allow for bystander effects in a fashion similar to TGF-β secreted by CD8^+ T cells in oral tolerance. As discussed in chapter one, there is evidence to suggest that antigen presentation by B cells may not only induce tolerance in naive T cells but also preferentially induce TH2 cells. In the Lewis rat model, EAE was suppressed when an encephalitogenic peptide was linked to anti IgD antibodies and thereby targeted to B cells for antigen presentation (Day et al, 1992). A relevant question to ask is
therefore whether Ac1-9 constitutes a sufficient epitope to be specifically taken up by B cells via membrane bound immunoglobulin. If this were the case then we should be able to raise antibodies against Ac1-9 after priming with this peptide alone. However, after priming with Ac1-9, only very few mice developed antibody responses to MBP or the longer peptide Ac1-20. In contrast, priming with Ac1-20 considerably increased the number of animals capable of mounting antibody responses to MBP or Ac1-20 (George Liu, unpublished data). A recently published report on a mouse model of allergic immune responses to the group I allergen Der p1 of the house dust mite showed that intranasal administration of the dominant CD4+ T cell epitope p1 111-139 prior to priming with the whole antigen Der p1 lead to profoundly diminished in vitro IL-2 production in response to both the dominant as well as other epitopes of Der p1. Although the question of bystander help was not addressed the authors argue in favour of classical suppression, since lymphocytes from mice treated by peptide inhalation were able to suppress in vitro antibody production by spleen cells from Der p1- primed mice (Hoyne et al, 1993). It is therefore tempting to speculate that this peptide p1 111-139 consisting of 18 residues was sufficiently long to serve as a B cell epitope thereby possibly enabling the induction of TH2-mediated suppression. Interestingly, however, this report also suggests a significant role for high avidity interactions of TCR-MHC-peptide interactions. While a single intranasal dose of the dominant epitopes was sufficient to inhibit in vitro T cell responses, at least three doses of a peptide containing a minor epitope were required to induce immune inhibition by inhalation.

Taken together, these finding suggest that high avidity ligation of the trimolecular complex under tolerogenic conditions (as provided by peptide inhalation) is central to the phenomenon of specific nonresponsiveness induced by intranasal administration of peptide. Although this implies an epitope-specific mode of inhibition, it is possible that additional suppressor mechanisms may be inducible with appropriate peptides, for instance if the peptide can serve as a B cell epitope. A direct comparison of Ac1-9 and Ac1-20 given by inhalation might provide further insight into possible B cell-mediated regulation in this model. However, even if Ac1-9 cannot efficiently bind to membrane bound immunoglobulin, this is not sufficient to disprove bystander regulation of the response to 35-47. More studies are needed to rigorously distinguish between the lack of bystander help and the presence of bystander suppression. As mentioned earlier, disrupting expression or recognition of Ac1-9 within MBP might shed more light on this issue.
5.3.2. v Can peptide inhalation affect the function of primed lymphocytes?

Without detailed knowledge of the mechanisms that are initiated after peptide inhalation, we will not be able to anticipate the biological consequences of intranasal inoculation of antigen under varying experimental conditions. We do know, however, that the inhibition of immune responses after peptide inhalation is not necessarily an all-or-none event and does not depend on the complete abrogation of T cell priming. In several experiments (e.g. figure 5.1 and figure 5.5 groups 1 and 2) the severity of EAE was markedly reduced while the incidence of disease was hardly affected. On a cellular level, these results may reflect successful or only moderately impaired priming and subsequent inhibition of effector functions rather than priming itself. Taking this issue further, we might then ask whether peptide inhalation could also inhibit effector functions after the induction of EAE. Apart from additional information about T cell regulation, this question is also of paramount importance for peptide inhalation as a potential therapeutic approach to various autoimmune and allergic conditions. Preliminary studies that address this point are presented in the following chapter.

5.4 SUMMARY AND CONCLUSION

The data presented in this chapter show that a single 100µg dose of Ac1-9 or Ac1-11 of MBP and its higher affinity analogues 4A and 4Y profoundly inhibit EAE when administered by inhalation one week prior to disease induction. The positive correlation between the degree of protection from EAE and the affinity of peptides for the H-2u molecule suggest that direct (CD8+ T-independent) interactions between APC and encephalitogenic T cells play a significant role for the intranasal route of tolerance induction. High avidity ligation of TCR-MHC-peptide complexes are possibly also important upon an antigenic challenge after peptide inhalation. This may explain the preliminary evidence that inhaled MBP 35-47 peptide failed to inhibit the weak in vitro response to this subdominant epitope after priming with SCH, while priming with 35-47 of MBP elicited strong specific in vitro responses which were inhibitable by prior inhalation of 35-47.

The finding that inhalation of a single peptide Ac1-9 or its higher affinity analogues protected against EAE induced with SCH may be explained by the large degree of dominance of Ac1-9 over other epitopes. Therefore, without the need for suppressor mechanisms, direct specific downregulation of the dominant response may be sufficient to inhibit autoimmune responses to the
whole antigen. However, inhibition of in vitro lymphocyte responses to 35-47 after 4A inhalation and priming with SCH suggested bystander effects (not shown). Since peptide inhalation prior to co-priming with a mixture of peptides revealed a strictly epitope-specific mode of inhibition, the bystander effect on the subdominant epitope after priming with SCH can be accounted for by a lack of bystander help that might otherwise be provided by the dominant immune response. However, bystander regulation within a tolerogenic microenvironment cannot be excluded.

Further studies are planned to investigate the route of peptide upon inhalation and the relevant location and APC for tolerance induction. A transgenic H-2^u mouse model of EAE will enable us to investigate the fate of encephalitogenic T cells after the inhalation of peptide or whole MBP and to facilitate transfer studies with potential regulator cells. On a cellular level, studies on T cell signalling may reveal physiological changes as a consequence of antigen encounter after inhalation.
**Figure 5.1** Inhalation Of Ac1-11 Before Peptide-Induced EAE

![Graph showing mean grade EAE over days after EAE induction]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9/9</td>
<td>14</td>
<td>4.1 ± 1.4</td>
</tr>
<tr>
<td>Ac1-11[4K]</td>
<td>6/8</td>
<td>16</td>
<td>1.7 ± 1.7</td>
</tr>
</tbody>
</table>

$^a P < 0.01$

**Figure 5.1** Pl/J mice inhaled 100µg Ac1-11 in a single 25µl droplet (closed circles) or 25µl PBS (open circles) under light ether anaesthesia. Seven days later, EAE was induced with 50µg Ac1-11/CFA.
Figure 5.2 Inhalation Of Ac1-11 And Ac1-11[4A] Before Peptide-Induced EAE

Figure 5.2 PL/J mice were treated with a single dose of 100µg Ac1-11[4K] (closed circles), Ac1-11[4A] (triangles) in 25 µl PBS or PBS alone (open circles) by inhalation. After one week, EAE was induced with 50 µg Ac1-11[4K]/CFA.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS</td>
<td>6/9</td>
<td>16</td>
<td>2.1 ± 1.9</td>
</tr>
<tr>
<td>2) Ac1-11[4K]</td>
<td>5/9</td>
<td>22</td>
<td>1.3 ± 1.9*</td>
</tr>
<tr>
<td>3) Ac1-11[4A]</td>
<td>2/10</td>
<td>&gt;time of experiment</td>
<td>0.2 ± 0.4*</td>
</tr>
</tbody>
</table>

<sup>a</sup> groups 1 and 3, p = 0.05
Figure 5.3 Effect of peptide - I-A^u affinity on protection from EAE by peptide inhalation

![Graph showing effect of peptide on protection from EAE](image)

### Table: Effect of Peptide Inhalation on EAE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE</th>
<th>MEDIAN DAY</th>
<th>MEAN MAXIMAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OF DISEASE^a</td>
<td>OF ONSET</td>
<td>GRADE EAE</td>
</tr>
<tr>
<td>1) PBS</td>
<td>9/10</td>
<td>13</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>2) Ac1-11[4K]</td>
<td>4/10</td>
<td>&gt;time of experiment</td>
<td>0.5 ± 0.7*</td>
</tr>
<tr>
<td>3) Ac1-11[4A]</td>
<td>2/10</td>
<td>&gt;time of experiment</td>
<td>0.2 ± 0.4*</td>
</tr>
<tr>
<td>4) Ac1-11[4Y]</td>
<td>0/10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

^a groups 1 and 2, *P* = 0.029
groups 1 and 3, *P* = 0.0027
groups 1 and 4, *P* = 0.00006

Figure 5.3 PL/J mice received a single intranasal dose of either 100µg Ac1-11[4K] (closed circles), Ac1-11[4A] (triangles), Ac1-11[4Y] (squares) or 25µl PBS alone (open circles) one week before EAE was induced with 50µg Ac1-11[4K]/CFA.
Figure 5.3 Effect of peptide - I-A\(^{u}\) affinity on protection from EAE by peptide inhalation

**Figure 5.3** PL/J mice received a single intranasal dose of either 100µg Ac1-11[4K] (closed circles), Ac1-11[4A] (triangles), Ac1-11[4Y] (squares) or 25µl PBS alone (open circles) one week before EAE was induced with 50µg Ac1-11[4K]/CFA.
Figure 5.4 Peptide Inhalation Before EAE Induced With Whole SCH In (PL/JxB10.PL) F1 Mice

![Graph showing mean grade EAE over time.]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9/9</td>
<td>17</td>
<td>2.5 ± 1.3</td>
</tr>
<tr>
<td>Ac1-11[4A]</td>
<td>2/10</td>
<td>&gt; time of experiment</td>
<td>0.7 ± 1.6*</td>
</tr>
</tbody>
</table>

<sup>a</sup> p = 0.0006

Figure 5.4 Mice were treated with 100µg Ac1-11[4A] (closed circles) or 25µl PBS (open circles) by inhalation. Seven days later disease was induced with 1mg SCH/CFA.
Figure 5.5 Effect Of Peptide Inhalation On EAE Induced With SCH In (PL/JxB10.PL)F1

Figure 5.5 Mice were given one intranasal dose of 100µg Ac1-9[4K] (closed circles), Ac1-11[4A] (triangles) or 25µl PBS only (open circles). One week later, EAE was induced with 1mg SCH/CFA.
Figure 5.6 Inhalation Of Ac1-11 And Its Higher Affinity Analogues Prior To SCH-Induced EAE In (PL/JXB10.PL)F1

Figure 5.6 Mice were given one intranasal dose of 100µg Ac1-11[4K] (closed circles), Ac1-11[4A] (triangles), Ac1-11[4Y] (squares), or 25µl PBS only (open circles). One week later, EAE was induced with 1mg SCH/CFA.
Figure 5.7 Inhalation Of Ac1-11 And Its Higher Affinity Analogues Prior To SCH-Induced EAE In B10.PL Mice

Figure 5.7 Mice inhaled 100μg of either peptide Ac1-11[4K], Ac1-11[4A], Ac1-11[4Y] or 25μl PBS one week before EAE was induced with 1mg SCH/CFA.
Figure 5.8 Inhalation Of Ac1-11 And Its Higher Affinity Analogues Prior To SCH-Induced EAE In PL/J mice

![Graph showing mean grade of EAE over time for different groups]

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET(^a)</th>
<th>MEAN MAXIMAL GRADE EAE(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9/10 + 1/10(^x)</td>
<td>13</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>Ac1-11 [4K]</td>
<td>8/10 + 2/10(^{xx})</td>
<td>42</td>
<td>2.4 ± 1.9</td>
</tr>
<tr>
<td>Ac1-11 [4A]</td>
<td>2/10 + 4/10 (^{xxx})</td>
<td>&gt;time of experiment</td>
<td>1.2 ± 2.0*</td>
</tr>
<tr>
<td>Ac1-11 [4Y]</td>
<td>2/10 + 3/10(^{xxxx})</td>
<td>&gt;time of experiment</td>
<td>0.4± 0.8*</td>
</tr>
</tbody>
</table>

\(^x\) indicate number of deaths without prior clinical signs of EAE.

\(^x\) day 36
\(^{xx}\) days 35 and 36
\(^{xxx}\) days 31-39
\(^{xxxx}\) days 16(1x) and day 36 (2x)

\(^a\) groups 1 and 2, \(p < 0.01\)
\(^b\) groups 1 and 2, \(p < 0.05\) (Behrens-Fisher test)

Figure 5.8 Mice inhaled 100\(\mu g\) of either peptide Ac1-11[4K], Ac1-11[4A], Ac1-11[4Y] or 25\(\mu l\) PBS one week before EAE was induced with 1mg SCH/CFA.
Figure 5.9 Effect Of Ac1-11[4Y] Inhalation On In Vitro Proliferation In Response To Ac1-11 And 35-47

PL/J mice inhaled 100 µg of Ac1-11[4Y] (closed symbols) or 25 µl PBS (open symbols). One week later they were immunized with either 50 µg of Ac1-11/CFA (circles) or 35-47/CFA (triangles). After 10 days, lymphocytes were restimulated in vitro with Ac1-11 (a) or 35-47 of MBP (b). Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Medium and PPD controls are arithmetic means and standard deviations of triplicate cultures in cpm.
Figure 5.10 Effect Of 35-47 Inhalation On In Vitro Proliferative Responses To Ac1-11 And 35-47

PL/J mice inhaled 100 µg of 35-47 (closed symbols) or 25 µl PBS (open symbols). One week later they were immunized with either 50 µg of Ac1-11/CFA (circles) or 35-47/CFA (triangles). After 10 days, lymphocytes were restimulated in vitro with Ac1-11 (a) or 35-47 of MBP(b). Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Controls are cpm arithmetic means ± SD of triplicates.
Figure 5.11 PL/J mice were immunized with 50 µg Ac1-11/CFA (circles), 50 µg 35-47/CFA (triangles) or 50 µg Ac1-11 plus 50 µg 35-47 of MBP in CFA included in the same adjuvant (squares). Ten days later, lymphocytes from draining lymph nodes were restimulated in vitro with Ac1-11 (a) or 35-47 (b). Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Medium and PPD controls are arithmetic means and standard deviations of triplicate cultures in cpm.

**Figure 5.11 Comparing The In Vitro Proliferative Response Of Lymphocytes After Priming With Ac1-11, 35-47, Or Co-Immunization With Both Peptides**
Figure 5.12 Effect Of Inhaling Either Ac1-9[4Y] Or MBP 35-47 On The Proliferative Response To Ac1-9 Or 35-47 After Co-Immunization With Both Peptides

Figure 5.12 PL/J mice inhaled 100 µg of Ac1-9[4Y] (closed circles) or 35-47 (closed triangles) or 25 µl PBS alone (open circles). One week later all were primed with both 50 µg Ac1-9 and 50 µg 35-47 in CFA included in the same adjuvant. Lymphocyte proliferation was assayed 10 days later in vitro against Ac1-9 (a) and 35-47 (b). Each symbol represents the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Medium and PPD controls are arithmetic means and standard deviations of triplicate cultures in cpm.
Figure 5.13 PL/J mice inhaled 100 µg of Ac1-9[4Y] (closed symbols) or 25µl PBS (open symbols). Seven days later, mice were primed with either 50µg Ac1-9[4K]/CFA (circles), 50µg MBP 35-47/CFA (triangles), or with a mixture containing 50µg of each peptide in CFA (squares). Lymphocyte proliferation was assayed 10 days later in vitro against Ac1-9 (a) and MBP 35-47 (b). Symbols represent the arithmetic mean of triplicate samples, standard deviations were less than 20% of the mean. Control values (means ± SD of triplicates) for medium and PPD are included in the legend.
CHAPTER 6

The Effect Of Peptide Inhalation On The Response Of Primed Encephalitogenic T Cells

6.1 INTRODUCTION

In the previous chapter we argued that the tolerogenic effect of the dominant MBP peptide Ac1-9 and its higher affinity analogues given by inhalation was largely governed by direct ligation of the T cell receptor under conditions that favoured nonresponsiveness over activation. This presumably involved modulation of T cell signalling in a yet undefined manner which might interfere with subsequent priming, thereby inhibiting the encephalitogenic potential of T cells. In this chapter we examine the consequences of peptide inhalation when T lymphocytes have already encountered myelin antigen after subcutaneous priming in complete Freund’s adjuvant. If T cells encounter further antigenic peptide (administered by inhalation) during an already ongoing immune response, three scenarios are conceivable:

1. A strong antigenic challenge with CFA irreversibly biases the immune response towards activation. If T cells are already stimulated to their maximum capacity, further antigen encountered at a later time point will not influence T cell functions any longer. At submaximal levels of T cell activation, inhaled peptide can only further stimulate but never inhibit an already ongoing immune response. In this situation we would expect mice treated with peptide by inhalation after priming either not to differ from control groups or to develop exacerbated disease.

2. Peptide inhalation under conditions shown to be tolerogenic before priming will also downregulate T cell activation after priming. This concept proposes that subcutaneous challenge of antigen in adjuvant and antigen
administration by inhalation initiate qualitatively distinct biochemical events within lymphocytes and perhaps also in APC. These mechanisms triggered by two modes of antigenic encounter will compete with each other and, depending on their relative strength, their effects may either cancel one another or one or the other may prevail. If this were true, then peptide inhalation after priming would be expected to have either no effect or to induce varying degrees of inhibition. We would not, however, expect exacerbation of EAE since an appropriate dose of peptide given by inhalation would always be tolerogenic by nature.

3. On a cellular level, the biochemical events following TCR-peptide-MHC ligation with inhaled peptide or peptide included in adjuvant do not substantially differ in quality. Rather, different quantities (thresholds) of the same or similar and interrelated intracellular events may determine qualitatively different T cell responses. This concept predicts that, depending on the timing between priming and inhalation and the resulting physiological state of immunocompetent cell, peptide inhalation after priming may initiate the whole spectrum of conceivable immunological effects: It may either exacerbate EAE, it may have no obvious consequences, or it may confer protection from disease.

All experiments presented in this chapter examine the effect of peptide inhalation after the induction of EAE with SCH. We aimed to administer a single intranasal dose of 100µg Ac1-9 or the 4A and 4Y analogues at a time point when T cells were already primed, yet before or around the onset of paralysis. After the onset of paralysis, further pathological signs of EAE may to some extent reflect T cell-independent inflammatory processes which might be difficult to manipulate any further in an antigen-specific manner. The onset of disease can vary among experiments. However, moderate body weight loss often precedes paralysis, presumably due to inflammatory mediators such as TNFα released by activated encephalitogenic T cells. The body weight was therefore monitored to anticipate the likely onset of EAE.

6.2 RESULTS

The results obtained from the first experiment (figure 6.1) indicate that inhalation of the 4A peptide 8 days after disease induction profoundly inhibited EAE. The plots of mean grade EAE and mean body weight are almost mirror images suggesting that weight loss and paralysis reflected disease severity in a similar fashion. The arrows marking the time points of peptide inhalation in the plots show that mice of both groups had lost some but very little weight
between days 3 and 8 after EAE induction. Nevertheless, one animal in the group that was to be treated with peptide already had a flaccid tail, so that peptide treatment was no longer postponed. Immediately after day 8 i.e. the day of peptide inhalation, the plots reveal rapid exacerbation of disease reflected both by rapid weight loss and grade of EAE in the control group. In contrast, animals that had received the 4A peptide by inhalation were substantially protected from further weight loss and paralysis. In place of the sharp peak of the first phase of disease in the control group, there were two smaller peaks in the 4A-treated group indicating both milder symptoms of EAE and an overall delay of disease onset (p< 0.05). Although the incidence of disease shows that most animals were not completely protected from EAE, the mean maximal grade was significantly lower in the peptide-treated group (p< 0.01). Both plots of figure 6.1 reveal that the protective effect of peptide inhalation was most pronounced during the first phase of EAE between days ten and twenty.

Our working hypothesis for subsequent experiments proposed that the earlier the time point chosen for peptide inhalation after EAE induction, the more readily will animals be protected from disease. To test this idea, four groups of mice received an intranasal dose of the 4A peptide or PBS either on day 8 or day 10 after EAE induction (figure 6.2). Appropriate timing for the earlier of the two time points was hampered by the finding that some mice were obviously still growing and gaining rather than losing weight (figure 6.2b). Nevertheless, day 8 proved to be the turning point followed by a steep rise in mean grade EAE (plots a and c) and rapid weight loss (plots b and d). In this experiment, inhalation of 4A on day 8 did not have such a pronounced effect as in the previous experiment (figure 6.1). However, even though the mean body weight was only marginally higher, mice treated with 4A displayed significantly milder grades of EAE than control animals (p=0.05). This was most obvious for the first phase of disease between days 10-20 (plot a) where 4A-treated mice had EAE of one grade lower on average compared with the control group. In contrast, inhalation of the 4A peptide two days later on day 10 no longer appeared to inhibit disease. Plot c of figure 6.2 shows an almost identical rise and climax of EAE around days 13-15 for both groups, although later on, 4A-treated mice seemed to have recovered more readily than control animals.

We then examined whether a protective “therapeutic” effect could also be achieved with the 4K and the 4Y peptides and -if yes- whether protection would be improved with the higher affinity analogue. Therefore, both peptides were included in the following experiment in which mice received an intranasal dose of either peptide 4K or 4Y or PBS eight days after disease induction (figure 6.3). The data clearly reveal that neither peptide inhibited EAE. Two explanations
could account for this result. Firstly, the protective properties of peptide inhaled after priming might be peculiar to the nonencephalitogenic 4A analogue. Secondly, the plots of figure 6.3 show that compared with the two previous experiments (figures 6.1 and 6.2), the average body weight had dropped more sharply by day 8, and while none of the mice displayed signs of paralysis on day 7, several animals in all groups had at least grade one EAE on day 8. It is therefore conceivable that day 8 in this experiment resembled the situation shown for day 10 in figure 6.2 where EAE was apparently already too advanced for inhaled peptide to confer protection.

Although the data presented in the table of figure 6.3 show virtually identical values for all groups, the differences in disease patterns among groups deserve further consideration. The course of EAE in the 4K-treated group seemed to closely follow the course of disease in the control group with a relatively mild first phase around days 10-14 and a second phase between days 25-30 with higher mean grades of EAE. In this case, the difference between the two phases was not reflected by the mean body weight for which the minimum coincided with the first phase of EAE and -after partial recovery- fell less sharply during the second phase. In contrast, mice that had inhaled the 4Y peptide got more severe EAE during the first phase compared with 4K-treated and control animals, but this was followed by faster recovery as shown both for the mean grade EAE and mean body weight. Although there were also some relapses between days 25-30 this was far below the degree of EAE observed for the other two groups during the same period and was not accompanied by weight loss.

These findings implied that the 4Y peptide could at least manipulate the course of disease. The question then arose as to whether inhalation of 4Y after EAE induction would not only ameliorate relapses (possibly at the expense of more severe EAE at an earlier stage) but could also interfere with the first phase of EAE provided it was administered early enough. The design of the experiment to address this question (figure 6.4) followed the same principle as shown for figure 6.2. We wanted to ensure that the earlier of the two time points lacked any signs of EAE either weight loss or paralysis. For figure 6.2 this was true for day 8. However, in the subsequent experiment (figure 6.3) disease was already more advanced by day 8. We therefore chose day 6 after EAE induction as the earlier of two time points for 4Y inhalation. Although not shown in the plots (figure 6.4 b and d), the body weight was also monitored daily between days 3 and 6. During that period there was no indication of weight loss. The plots of figure 6.4 show that the two time points, days 6 and 8 appeared to be appropriate for the purpose of our question: day 6 as an early post priming-pre EAE stage and day 8 already indicating a steep fall in body weight and
subsequent rapid onset of disease. The plots of figure 6.4 reveal that the effect of 4Y inhalation as a function of time after the induction of EAE was somewhat contrary to our working hypothesis. 4Y administered on day 8 was more protective than on day 6, the opposite of what we would have expected. For the first phase of EAE between days 10 and 15, 4Y inhaled on day 6 appeared to evoke more vigorous disease compared to the control group (figure 6.4a and b). The almost complete recovery in both groups was succeeded by a second peak of virtually the same shape as the first peak in the control group while this second phase of EAE between days 20 and 30 was substantially dampened in the 4Y-treated group (figure 6.4a). In this experiment there was also a third wave of disease between days 38 and 50 among controls. In contrast, none of the 4Y-treated mice displayed further signs of paralysis at this late stage. In summary, the overall effect of 4Y inhalation on day 6 was a somewhat aggravated first phase of disease compared to controls after which 4Y-treated mice were largely protected from the pronounced relapses characteristic of the control group in this experiment (figure 6.4a and b).

The development of disease after the inhalation of 4Y on day 8 disclosed a very different picture (figure 6.4c and d). Between days 6 and 8, the body weight plots for both the controls and the group that was to be treated with 4Y were virtually identical. On average, mice lost almost two grams, i.e. about ten percent of their body weight between days 6 and 8. While the average weight dropped continuously in the control group reaching its minimum on day 16, the 4Y-treated group showed only marginal further weight loss between days 8 and 9 followed by a short period of rapid recovery until on day 11, the original level of weight was reached again. Thereafter, a shallow trough appeared to parallel the weight loss in the control group, albeit on a much smaller scale. The protective effect of 4Y inhalation on day 8 was also clearly revealed by substantially diminished disease severity. The values in the table indicate that although most mice were not completely protected from EAE, the overall maximal grade of EAE was significantly lower for 4Y-treated animals (p<0.02). Furthermore, figure 6.4c illustrates that in contrast to controls, there were hardly any relapses between days 35 and 50. In addition, 4Y-treated mice tended to develop EAE more slowly than controls. Although the values in the table do not state a statistically significant delay, the slope of mean grade EAE in figure 6.4c is steeper for controls than for the 4Y group, reaching its maximum on day 16 for controls and on day 23 for the 4Y-treated group. Thus, in contrast to 4Y inhalation on day 6, treatment with this peptide by inhalation two days later on day 8 inhibited EAE during the whole course of this experiment.
We wished to confirm the observation that the 4Y peptide administered by inhalation at an early time point after disease induction tended to exacerbate the first phase of EAE while protecting against relapses. Unlike with 4Y, figure 6.2 suggests that the idea of better protection at an earlier time point seems to hold true for the 4A peptide. Perhaps if 4A were inhaled even before day 8 we would observe still more pronounced inhibition of EAE. Therefore, in the following experiment (figure 6.5), the effect of all three peptides 4K, 4A, and 4Y given by inhalation on day 6 after EAE induction was compared. Both plots of figure 6.5 show that the disease profile of the 4Y group differs most clearly from the controls and the two other peptide-treated groups. The first phase of EAE in the 4Y-treated group is characterized by a steep, narrow peak revealing more rapid and -at its maximum- more severe EAE than any of the other groups. Again, as observed in previous experiments, mice that had inhaled 4Y appeared to be protected from relapses. These observations are also illustrated by the plot of mean body weight showing the sharpest fall in weight ensued by the highest weight gain until the end of the experiment.

The effects of the other two peptides 4A and 4K were less clear. Inhalation of 4A on day 6 certainly did not confer the degree of protection we might have predicted from previous data. In fact, the plots for the 4A and control groups are so similar that any emphasis on marginal differences would presumably be misleading. The 4K peptide, however, might have supported the development of EAE in this experiment. There was a tendency (although not significant) of higher mean maximal grades of EAE after 4K inhalation. The plot of mean grade EAE shows a slightly earlier onset in the 4K group then in all other groups. Furthermore, fewer 4K-treated mice recovered after the first phase of disease and more animals relapsed compared to all other groups.

6.3 DISCUSSION

Among the three proposed outcomes of peptide inhalation after the induction of EAE, no effect/further activation, no net effect/inhibition, or either no effect/inhibition/further activation, the data presented in this chapter are only consistent with the third hypothesis. Sometimes, the course of EAE after peptide inhalation closely resembled disease in the control group, while in other experiments or with other peptides, inhalation clearly reduced disease severity or modified the course of EAE to a more pronounced first phase followed by an apparent resistance to relapses. Thus, even 6-10 days after a strong antigenic and inflammatory challenge such as with SCH in CFA, primed encephalitogenic T
cells were not irreversibly locked into a certain activation pathway but remained susceptible to modification by intranasally administered peptide.

Having rejected the concept of a characteristic biochemical signalling pathway that induces T cell nonresponsiveness as a tolerogenic default mechanism after peptide inhalation, it is likely that the effect of inhaled peptide is largely determined by the physiological state of specific T cells. Furthermore, while 100μg of peptide was suitable to downregulate a presumably small number of specific T cells before priming (chapter 5), after EAE induction, the same amount of intranasally administered peptide might not necessarily be sufficient to downregulate an expanding population of encephalitogenic T cells. Prior to an antigenic challenge in CFA, peptide inhalation consistently induced downregulation of in vivo and in vitro responses of Ac1-9 specific T cells, as shown in chapter 5. After priming, the effects of inhaled peptide appeared to cover the whole spectrum of conceivable events, inhibition, no net effect, or exacerbation of EAE. In order to predict the effect of peptide inhalation after priming, it will therefore be essential to characterize the physiological state of T cells at various time points after priming. Without detailed knowledge of intracellular events and the surface expression of certain activation markers (e.g. characteristic isoforms of CD45), this remains largely speculative. Initially, we proposed that peptide inhalation at earlier time points would inhibit EAE more readily than at a later stage. This idea implies a linear increase in T cell activation (both with respect to absolute numbers, i.e. proliferation of precursors, and the activation state of individual T lymphocytes) after priming and, in parallel, a linear decrease in the susceptibility to tolerance induction.

However, the data reveal a more complex picture than we would have expected according to this simple rule. In figure 6.1, inhalation of 4A 8 days after EAE induction profoundly inhibited the development of disease. The degree of protection was similar to what we had previously observed for 4A inhalation before the induction of EAE (figures 5.5 and 5.6). Yet, in another experiment in which mice received an intranasal dose of 4A two days earlier on day 6 (figure 6.5) both the plot and the data in the table show virtually identical patterns of disease between 4A-treated and control groups. Although it is difficult to draw conclusions about timing between two separate experiments, there was no indication of an unusually rapid onset of disease or weight loss in the experiment of figure 6.5. It is therefore unlikely that the lack of protection by 4A inhalation on day 6 in this experiments as opposed to day 8 in figure 6.1 can be explained by a higher degree of T cell activation at which 4A inhalation could no longer be protective. In a direct comparison of 4A inhalation on days 8 and 10, there was some indication for protection after 4A inhalation on day 8 (figure
6.2a) although the effect on weight loss was marginal (figure 6.2b). Two days later on day 10, the peak of disease between controls and 4A-treated animals were virtually identical. The significance of the more complete recovery of 4A-treated mice was less clear since the overall weight gain was similar in both groups (figure 6.2d), although more mice remained with chronic low grade EAE in the control group (figure 6.2c). Two interpretations of these findings are possible: the prediction of better protection at an earlier time point after EAE induction might have been true for this experiment. Alternatively, if the more rapid recovery of 4A-treated mice on day 10 were significant, then 4A inhalation on day 10 was too late to affect the peak of disease, but at later stages, it supported recovery from disease. On a cellular level, 4A might have accelerated the downregulation of activated T cells after the first phase of EAE.

A direct comparison of the effect mediated by the highest and lowest affinity peptides 4K and 4Y administered 8 days after EAE induction provides further insight into possible modulation of T cell activity by inhaled peptide (figure 6.3). 4Y inhalation caused a strikingly different pattern of disease, with a steep higher first peak of EAE followed by a very low grade relapse compared to both control and 4K-treated groups. This modification of the course of EAE by 4Y was confirmed in a direct comparison with 4K and 4A inhalation on day 6 (figure 6.5) and also when tested on its own against controls in another experiment shown in figure 6.4 a and b. This pattern of an exacerbated first phase after which animals were more protected from further relapses was characteristic after inhalation of 4Y and not observed with either 4K or 4A in any experiment. Presumably, this phenomenon reflects an additive effect of 4Y to T cell priming after which encephalitogenic T cells were either no longer present or refractory to further stimulation. It is tempting to compare this finding with the phenomenon of anergy or deletion through activation discussed in chapter 1. Here, high doses of soluble antigens in the periphery induce cell death or T cell anergy, often preceded by a phase of activation. A recent study investigated the phenomenon of high dose tolerance in a transgenic H-2u mouse model of EAE, with transgenic TCR specific for Ac1-11 of MBP. High intravenous doses of MBP (8x400µg) or Ac1-11 (8x1.4mg) caused deletion of encephalitogenic T cells and protected mice from disease. On a cellular level, this finding was explained by TCR reengagement after initial T cell activation and cell cycle progression at high levels of IL-2, conditions required to induce cell death in vitro (Critchfield et al, 1994). Although the experimental conditions for our studies were different, inhalation of 4Y after EAE induction possibly met the conditions for this form of high zone tolerance. Priming with SCH in CFA represents a strong antigenic challenge that probably induces high levels of IL-2. Presumably, inhalation of 4Y
initially accelerated and supported T cell activation and IL-2 production even further as suggested by steeper and higher peaks of EAE compared to controls. This might have lead to cytotoxic lymphokine levels that subsequently either induced cell death or T cell anergy. Although the amounts of peptide used to induce high zone tolerance in the report by Critchfield et al were about 100 times higher than the intranasal dose of peptide administered in our experiments, it is possible that the ability of 4Y to form stable complexes with I-Au was sufficient to reach the threshold of TCR-MHC-peptide engagement required for high zone tolerance. If this were true, then perhaps high zone tolerance reflects high avidity rather than just high dose tolerance. The finding that the same amount (100µg) of neither 4K or 4A given by inhalation induced the same effect is consistent with this idea (figures 6.3 and 6.5). In this case, higher intranasal doses of 4K and 4A might be able to compensate for the lack of affinity.

However, plots c and d of figure 6.4 show that the effect of 4Y did not always follow the same pattern of early disease exacerbation and subsequent inhibition. Here, 4Y inhalation 8 days after EAE induction reduced the severity of disease over the whole course of the experiment. Possibly at this later stage (compared to day 6 in the same experiment) T cell activation had passed its maximum already so that TCR ligation with further antigen only caused anergy or deletion, but no longer further activation. This may also account for the high degree of protection achieved by 4A inhalation shown in figure 6.1. The ability of 4A to protect might either be due to a very early stage of T cell activation at which T cells were still susceptible to tolerance induction, or, alternatively, T cells might have passed a physiological stage of maximal cell cycling and IL-2 production after which they were again susceptible to downregulation. However, in this case we have to conclude that the loss of body weight did not faithfully reflect T cell activation simultaneously, but occurred with some delay after maximal activation. However, while this idea is consistent with the data of figures 6.1, 6.4 and 6.5, it does not account for the results in figure 6.3 showing the characteristic pattern of enhanced disease/inhibition after inhalation at a time when the average body weight had already fallen dramatically. Clearly, without further kinetic studies, including a direct comparison of all three peptides as well as a biochemical analysis of T cell at different times after disease induction, all explanations will be largely speculative. However, the data permit one conclusion: there appeared to be at least two distinct stages at which peptides given by inhalation exerted different effects. At one stage (perhaps a stage of maximal activation) inhalation of 4Y lead to an enhanced first peak of disease followed by profound protection from further relapses (figures 6.3-6.5). Under these conditions, neither the 4A or the 4K-treated groups displayed a disease
pattern that differed substantially from controls (figures 6.3 and 6.5). At another stage, inhalation of 4A inhibited disease to some extent (figures 6.1 and 6.2a), and inhalation of 4Y at this same or yet another stage of T cell priming conferred protection throughout the whole course of EAE (figure 6.4c).

6.4 SUMMARY AND CONCLUSION

Modulation of encephalitogenic T cells by peptide inhalation 6-10 days after EAE induction with SCH was possible but the effect appeared largely unpredictable. The positive correlation between peptide-I-A\(^u\) affinity and the degree of protection from EAE as shown in chapter 5 for inhalation as a pre-treatment did not apply to peptide inhalation after disease induction. Instead, different peptides seemed to modulate EAE in different ways: 4K had either no effect or exacerbated disease slightly. 4A had either no effect or inhibited EAE to varying degrees between different experiments. Treatment with 4Y induced a characteristic pattern of an exacerbated first phase of disease after which animals were refractory to further relapses. This phenomenon resembles the concept of anergy/deletion through activation described for several models of peripheral tolerance. In one experiment, however, the effect of 4Y was different and reduced disease severity during the whole course of the experiment. These findings imply differential effects of intranasally administered peptide on different stages of T cell activation. It will now be necessary to analyze the effect of peptide inhalation on T cell physiology at different stages of activation. Additionally, different experimental conditions such as repeated rather than single doses of inhaled peptide might be required to override T cell activation in favour of tolerance induction.
Figure 6.1 (B10.PL x PL/J) F1 mice were primed with 1 mg SCH/CFA for the induction of EAE. Eight days later, all were treated with 100 µg Ac1-11[4A] in PBS (closed circles) or 25 µl PBS alone (open circles) by inhalation.

Figure 6.1 Inhalation of Ac1-11[4A] eight days after the induction of EAE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAEa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10/10</td>
<td>11.5</td>
<td>2.9 ± 1.1</td>
</tr>
<tr>
<td>Ac1-11[4A]</td>
<td>7/10</td>
<td>19.5</td>
<td>1.2 ± 1.0</td>
</tr>
</tbody>
</table>

a p<0.05  b p<0.01
Figure 6.2 (B10.PL x PL/J) F1 mice were primed with 1 mg SCH/CFA for the induction of EAE. All mice inhaled either 100 µg Ac1-11[4A] (closed circles) or 25 µl PBS (open circles) either 8 days (a, b) or ten days (c, d) after disease induction.

**Figure 6.2** Inhalation of Ac1-11[4A] on days eight or ten after the induction of EAE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS, d8</td>
<td>10/10</td>
<td>12</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>2) Ac1-11[4A], d8</td>
<td>9/9</td>
<td>14</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>3) PBS, d10</td>
<td>10/10</td>
<td>12</td>
<td>2.7 ± 1.5</td>
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<tr>
<td>4) Ac1-11[4A], d10</td>
<td>10/10</td>
<td>12</td>
<td>2.6 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup><sub>p = 0.05 for 1 versus 2</sub>
Figure 6.3 Inhalation of Ac1-9[4K] or [4Y] eight days after the induction of EAE

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS</td>
<td>10/10</td>
<td>11.5</td>
<td>2.7 ± 1.5</td>
</tr>
<tr>
<td>2) Ac1-9[4K]</td>
<td>10/10</td>
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<td>2.9 ± 1.4</td>
</tr>
<tr>
<td>3) Ac1-9[4Y]</td>
<td>10/10</td>
<td>11.0</td>
<td>2.7 ± 0.9</td>
</tr>
</tbody>
</table>

Figure 6. (B10.PL x PL/J) F1 mice were primed with 1 mg SCH /CFA for the induction of EAE. Eight days later, all received an intranasal 100 µg dose of either Ac1-9[4K] (closed circles), Ac1-9[4Y] (closed triangles), or 25 µl PBS alone (open circles).
Figure 6.4 (B10.PL x PL/J) F1 mice were primed with 1 mg SCH/CFA for the induction of EAE. Mice received an intranasal dose of 100 µg Ac1-9[4Y] (closed circles) or 25 µl PBS (open circles) on day six (a, b) or day eight (c, d) after the induction of EAE.

Figure 6.4 Inhalation of Ac1-9[4Y] on day six or day eight after the induction of EAE

| GROUP | INCIDENCE OF DISEASE | MEDIAN DAY OF ONSET | MEAN MAXIMAL GRADE EAE*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS, d6</td>
<td>8/9</td>
<td>11</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>2) Ac1-11[4Y], d6</td>
<td>9/9</td>
<td>11</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>3) PBS, d8</td>
<td>8/9</td>
<td>13</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>4) Ac1-11[4Y], d8</td>
<td>6/9</td>
<td>17</td>
<td>1.3 ± 1.2</td>
</tr>
</tbody>
</table>

*p < 0.02 for 3 versus 4
Figure 6.5 (B10.PL x PL/J) F1 mice were primed with 1 mg SCH/CFA for the induction of EAE. Six days later, all were treated with 100 µg of either Ac1-9[4K], Ac1-9[4A], Ac1-9[4Y] or 25 µl PBS by inhalation.

Figure 6.5 Comparing the effect of Ac1-9[4K], [4A], and [4Y] given by inhalation six days after the induction of EAE.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INCIDENCE OF DISEASE</th>
<th>MEDIAN DAY OF ONSET</th>
<th>MEAN MAXIMAL GRADE EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) PBS</td>
<td>6/9</td>
<td>13</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>2) Ac1-9 [4K]</td>
<td>7/10</td>
<td>12.5</td>
<td>2.0 ± 1.4</td>
</tr>
<tr>
<td>3) Ac1-9 [4A]</td>
<td>7/10</td>
<td>12.5</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>4) Ac1-9 [4Y]</td>
<td>7/9</td>
<td>12</td>
<td>1.3 ± 1.1</td>
</tr>
</tbody>
</table>
7.1 ACTIVATION VERSUS TOLERANCE BY ANTIGEN ADMINISTRATION VIA MUCOSAL SURFACES

The intragastric and intranasal routes of antigen administration have been widely explored to achieve two opposite effects of antigen on the immune system: specific activation and tolerance. Both routes have been studied for the induction of anti-viral immune responses to develop suitable strategies for vaccination (Van Cott et al., 1993; Onorato et al., 1991), and a wide range of effector mechanisms, many of them IgA- and IgE-mediated humoral responses combat bacterial and parasite infections in the intestinal- and respiratory tract (Mazanec et al., 1993; Hagan, 1993). In contrast, as described in numerous previous reports and shown in this thesis, intragastric and intranasal administration of soluble antigens often induces a state of specific immunological nonresponsiveness.

In order to develop appropriate immune responses to either potentially detrimental pathogens or innocuous agents, safe and unambiguous criteria are required to guide the immune system towards activation or tolerance. Similar to their effect on priming by subcutaneous administration, bacterial particles appear to favour the development of effector functions as opposed to tolerance after oral or intranasal antigen administration. Oral tolerance was more readily demonstrated with soluble proteins than with particulate bacterial antigens, probably due to an adjuvant effect of bacterial lipids (Challacombe & Tomasi, 1980). However, feeding particulate antigens may also induce oral tolerance as shown for sheep red blood cells (Mattingly & Waksman, 1978), suggesting that the absence of bacterial components is perhaps more important than the solubility of an antigen. Curiously, however, bacterial LPS, commonly used as a nonspecific B cell activator, appeared to support the induction of oral tolerance.
in the Lewis rat model of EAE (Khoury et al, 1990). In this case, a later report suggested that the local adjuvant effects of LPS in GALT resulted in the activation of PGE$_2$-producing regulator cells which synergized with TGF-β secreting CD8$^+$ T cells to protect rats from EAE (Khoury, Hancock, & Weiner, 1992).

As mentioned earlier, alveolar macrophages (AM) create an immunosuppressive environment in the respiratory tract by means of their apparently inherent ability to inhibit T cell responses directly, and to functionally impair the ability of airway dendritic cells to activate T cells. However, under experimental conditions that mimic a challenge with inhaled microbial (and thus potentially pathogenic) antigens, AMs lose their immunosuppressive capacity. In one study, AMs incubated with medium generated with LPS-stimulated whole lung, no longer inhibited in vitro lymphocyte proliferation. Therefore, in this situation, LPS inhibited rather than supported immunosuppression. This effect could be abrogated with antibodies to granulocyte/macrophage colony-stimulating factor (GM-CSF), mimicked with recombinant GM-CSF, and enhanced by TNFα (Bilyk and Holt, 1993).

An adjuvant effect was also achieved when antigens were incorporated into synthetic lipid particles (lipophilic immune-stimulating complexes, ISCOMS). In these studies, oral administrations of antigens such as OVA which usually induce oral tolerance when given in a soluble form, provoked a wide range of local and systemic immune responses upon feeding in ISCOMS. Such lipid particles also functioned as adjuvants for intranasally applied antigens (reviewed in Mowat & Donachie, 1991). Bacterial toxins such as pertussis and cholera toxins also served as adjuvants for orally administered antigens and abrogated tolerance induction by antigen feeding or inhalation (reviewed in Holt & McMenamin, 1989; Pierre, Denis, & Bazin, 1992). Together, these reports suggest that generally, antigens administered by feeding or inhalation only induce tolerance if they are encountered in the absence of substances with adjuvant properties. LPS might represent an exception to this rules for models of oral tolerance in which LPS activates regulator cells that support the inhibitory effect of fed antigen.

However, even in the absence of adjuvants, soluble antigens given by feeding or inhalation do not induce nonresponsiveness by default. As discussed before, the dose and timing constitute significant parameters for tolerance induction. In the OVA model of oral tolerance, doses between 1-25 mg suppressed subsequently induced systemic immune responses, while some smaller doses had either no effect or supported priming (Lamont, Mowat, & Parrot, 1989). In studies on oral tolerance in models of autoimmune diseases, there was either a
positive correlation between the amounts of fed antigen and tolerance (Thompson et al, 1993; Nussenblatt et al, 1990; Higgins & Weiner, 1988) or, by contrast, smaller doses were more effective than high oral doses (Thompson & Staines, 1985; Zhang et al, 1990). Generally, repeated feeding tended to induce tolerance more readily than a single dose (Ngan & Kind, 1978; Higgins & Weiner, 1988).

Although a thorough analysis of these factors, dose and timing, was not central to this thesis, some of the data presented and other preliminary observations allow for some comments. In chapter 3, feeding 3 x 7 mg OVA 7, 5, and 3 days before priming (figure 3.9) reduced T cell proliferation to at least the same degree as a single 20 mg dose given 7 days before an antigenic challenge. Compared to some experiments (e.g. figures 3.4 and 3.6), 3 x 7 mg appeared to be more effective than 1 x 20 mg. However, in other experiments in which mice received a single 20 mg dose, the titration plots of lymphocytes from OVA-fed mice were similar to the data of figure 3.9 (figures 3.1 and 3.5) so that the benefit of repeated lower doses of OVA was presumably not significant. Other preliminary studies in the Balb/c model that addressed the dose requirement for orally administered OVA to affect T cell proliferation suggested that 3 x 1 mg OVA given 7, 5, and 3 days before priming also resulted in lower proliferative responses (not shown). In contrast, the data presented in chapter 4 reveal that, with the same doses and similar timing (3 x 1 mg on days -7, -5, -2), MBP did not induce oral tolerance in the H-2u mouse model as assessed by the failure to protect against EAE. In some experiments, oral administration of Ac1-11 or MBP even tended to exacerbate disease at some stages (discussed in chapter 4).

Fewer reports are available on dose-responses for intranasal administration of antigen. In the mouse model of immunity to the house dust mite antigen Der P1, in vitro T cell nonresponsiveness was equally induced by either of 3x 1µg, 10µg, or 100 µg of Der P1 or the peptide representing a dominant epitope (Hoyne et al, 1993). In the Lewis rat model of EAU, intranasal doses of retinal extract ranging form 4.2mg-4.2µg per day for 10 days appeared to inhibit disease to a similar extent. Below that dose, rats were less well protected (Dick et al, 1993). In the H-2u mouse model of EAE, a single intranasal dose of 100µg Ac1-11 or the 4A and 4Y analogues administered 7 days before immunization was appropriate to inhibit EAE and -as shown for 4Y- to abrogate in vitro T cell proliferation (chapter 5). However, preliminary experiments applying the same protocol to other models provided no evidence for tolerance. A single dose of 100µg OVA 323-339 or 2 mg OVA administered intranasally did not reduce T cell proliferation in response to OVA (not shown). Preliminary attempts to induce tolerance by intranasal administration of encephalitogenic peptides in
another mouse model of EAE were unsuccessful (David Wraith, unpublished observations). Therefore, even in the absence of adjuvants, an effective dose (and appropriate timing) capable of inducing tolerance by the oral or intranasal route appears to be characteristic for each antigen and experimental model.

### 7.2 MUCOSAL VERSUS PERIPHERAL MECHANISMS OF TOLERANCE

#### 7.2.1 Oral Administration Of Antigen

Orally and intranasally administered antigens are encountered by lymphoid elements in the intestinal and respiratory tract, but they are also absorbed into the peripheral circulation, thus adding some complexity to attempts to evaluate the relative significance of local mucosal versus general peripheral contributions to tolerance induction.

In oral tolerance to OVA, studies on the tolerogenic serum factor (TF) suggested that both the generation and action of TF might be independent of GALT (discussed in chapter 3). If TF induced regulator cells as concluded from the ability to abrogate tolerance in serum recipients by CY-treatment, then these putative suppressor cells were also GALT-independent. On the other hand, transfer of spleen cells but not OVA-fed serum inhibited subsequent antibody responses in recipients. Together, these observations suggest that oral administration of OVA generates more than one type of regulator mechanism, one of which is GALT-independent and induced by TF, but confined to cellular immune responses such as DTH, whereas direct feeding may induce regulator cells that are present in the spleen seven days after feeding, and which are also capable of downregulating humoral immune responses. In contrast, neither spleen cell transfer from donors fed with OVA one week before nor transfer of OVA-fed serum (containing both TF and SF) by the i.v. or i.p. route could substitute for direct oral administration of OVA to inhibit secondary in vitro T cell proliferation. In chapter 3, we argue that the failure to reduce T cell proliferation by feeding OVA peptide 323-339 alone is not easily reconcilable with a concept of direct T cell anergy as the only mechanism for this in vitro phenomenon of oral tolerance. If the preliminary data obtained from the in vivo CD8 depletion experiment hold up, then CD8+ regulator cells might not be involved at early stages after OVA feeding. However, the ability to show some inhibitory effect on T cell proliferation by the transfer of MLN cells 2-4 days after OVA feeding implies a role for an early GALT-derived regulatory cell
(Challacombe & Tomasi, 1980). These findings together with results presented in chapter 3 and other reports that failed to provide evidence for suppression in vitro or upon spleen cell transfer suggest that the in vitro phenomenon of reduced T cell proliferation after OVA-feeding might reflect the outcome of a GALT-dependent regulatory mechanism that takes place at an early time point after feeding. 

Although TCRγδ+ T cells represent a predominant population of intraepithelial lymphocytes in the gut, their strikingly different mode of maturation, antigen recognition, and MHC restriction compared to TCRαβ+ T cells (discussed in chapter 1) makes them unlikely candidates for suppressor cells in oral tolerance. To our knowledge, there is only evidence for a role of CD4+ or CD8+ TCRαβ+ regulator cells in oral tolerance. These may arise from GALT, but presumably from PP and/or MLN rather than the gut epithelium. Transfer of PP- or spleen cells from OVA-fed donors inhibited OVA-specific IgE and IgG titres in recipients. (Ngan & Kind, 1978). Oral tolerance to SRBC inhibited both the number of plaque forming cells (PFC) and DTH responses in fed rats. In this model, the significance of suppressor cells was demonstrated by the ability of PP-, MLN-, spleen- or thymic cells from SRBC-fed donors to reduce the number of PFC in vitro. Kinetic studies suggested a migration pattern of suppressor cells form GALT to peripheral lymphoid tissues and to the thymus. Two days after feeding, suppression of PFC was observed with cells form PP and MLN, but not with thymic or spleen cells. In contrast, 4 days after feeding, suppressor cells were present in the spleen and thymus, but no longer in PP and MLN (Mattingly & Waksman, 1978). As discussed in chapter 4, oral tolerance in the Lewis rat model of EAE was attributed to CD8+ T cells in mesenteric lymph nodes and spleens from MBP-fed animals which conferred protection from EAE in recipients, and inhibited proliferative responses in vitro (Lider et al, 1989). Subsequent studies revealed TGF-β as a major inhibitory lymphokine to account for specific suppression as well as for the ability to mediate suppression to a second antigen in a bystander fashion (Miller, Lider & Weiner, 1991; Miller et al, 1992a). Similarly, oral tolerance to the hapten trinitrochlorobenzene (TNCB) in mice was also mediated by suppressor cells in GALT and spleen that produced a nonspecific soluble suppressor factor upon TNCB-specific activation. However, this form of immune regulation was not confined to antigen feeding but was also observed after i.v. injection of TNCB (Gautam & Battisto, 1985). Furthermore, the dose dependence of orally administered antigen to induce either suppressor-mediated regulation at low doses or suppressor-independent tolerance at high doses (Gregerson, Obritsch, & Donoso, 1993, discussed in chapter 4) was observed as early as 1979 in elegant studies by Sherr et al for i.v. injected antigen
coupled to splenocytes. In these experiments, i.v. injection of 1ng of a palmitoyl derivative of fowl γ-globulin (p-FγG) coupled to 10^3 spleen cells induced a form of tolerance that was transferable after a minimum of 7 days and could be abrogated by CY-treatment. Analogous to what was later referred to as bystander suppression, recipients of spleen cells from FγG-nonresponsive mice (as assessed by DTH to FγG and PFC responses to TNP-FγG) also showed suppressed TNP-specific responses when immunized with TNP-KLH provided that FγG was added to the inoculum, thereby demonstrating that the activity of FγG-induced suppressor cells was non-specific but not manifest unless the suppressor cells were exposed to the antigen used for their induction. Alternatively, at least 10^6 antigen-coupled splenocytes were required to induce nonresponsiveness at an early stage 3 days after i.v. injection. Under these conditions, tolerance could not be transferred and was not inhibited by CY-treatment, suggesting a direct, Ts-independent mechanism (Sherr et al, 1979). Together, these studies suggest that the induction of a suppressor mechanism characterized by low dose stimulation and a nonspecific effector component may ensue from but is not restricted to the intragastric route of antigen administration.

However, while in some models, high dose feeding prevented the induction of suppressor cells, this did not seem to apply to the mouse model of oral tolerance to OVA. An oral dose of 20 mg OVA can probably be regarded as high. Yet, as discussed in chapter 3, feeding this dose was appropriate for the induction of regulator cells as well as for phenomena of T cell nonresponsiveness consistent with anergy. It is therefore possible that in this model, doses of orally administered antigen suitable to induce either suppression or anergy overlap, so that both mechanisms can be induced under the same experimental conditions.

Although orally administered antigen may lead to T cell anergy under some conditions, it is not clear whether anergy is induced within GALT or in peripheral lymphoid organs upon antigen absorption into the general circulation. The only successful attempt to induce oral tolerance to EAE in PL/J mice was made with high doses (3x1mg) of the highest affinity analogue Ac1-9[4Y] (chapter 4). This did not prove but was consistent with the induction of T cell anergy favoured by high avidity TCR-MHC-peptide interactions. In chapter 4 we argue that with these high oral doses of a single peptide, it is likely that a considerable amount of peptide gained access to the periphery, although this does not exclude that encephalitogenic T cells were also rendered nonresponsive within GALT. However, if high avidity interaction of the trimolecular complex were a sufficient criterion for anergy induction, then it would be difficult to
understand why the same amounts of orally administered OVA 323-339 peptide failed to downregulate T cell proliferation (chapter 3). This peptide was shown to form stable complexes with I-A\(^d\) (Buus et al, 1987), and should therefore fulfill the requirement to mediate high avidity interactions. The strong proliferative responses to OVA 323-339 exclude the possibility of inefficient TCR interactions with OVA 323-339-I-A\(^d\) complexes. However, this peptide also failed to induce nonresponsiveness by inhalation (not shown), in sharp contrast to Acl-9 and its analogues (chapter 5). One might speculate that T cells specific for Acl-9 and OVA 323-339 somehow differ in their biochemical make-up even before an encounter with their respective antigens in the periphery. As discussed in chapter 1, Acl-9 might represent a naturally processed epitope which, as part of the trimolecular TCR-MHC-peptide complex, does not reach the avidity threshold for negative selection. In this case, although T cells specific for Acl-9 escape deletion in the thymus, they might nevertheless be biochemically "stamped" by a cognate antigenic encounter within the thymus. As a consequence, they might be more readily downregulated as mature T cells than those T cells with specificity for non-self molecules such as OVA 323-339 that were not presented for negative selection.

To conclude, oral tolerance does not appear to operate by mechanisms that are confined to GALT, and that could not principally also be induced by peripheral antigen administration in some models under appropriate experimental conditions. This does not exclude, however, that in certain models such as oral tolerance to OVA, the oral route of antigen administration may induce tolerance more efficiently and/or a wider range of mechanisms than could be achieved by a peripheral encounter of antigen alone.

### 7.2.2 Intranasal Administration Of Peptide

The data presented in chapter 5 show a positive correlation between the affinity of intranasally applied peptide (Acl-9 or 1-11 and the 4A and 4Y analogues) and the degree of protection from EAE. Bystander effects to the subdominant epitope MBP 35-47 after priming with SCH are possible. However, the above finding suggests that initially, a direct (suppressor-independent) tolerogenic effect on encephalitogenic T cells favoured by high avidity TCR-MHC-peptide interactions plays a predominant role in this model. The underlying mechanism might therefore largely operate by T cell anergy, and possibly also by deletion. As discussed in chapter 1, anergy and deletion were
frequently observed after peripheral antigen administration (i.v. or i.p.) in a tolerogenic form (soluble, or in IFA, or coupled to fixed APCs so as to abrogate costimulation). On the other hand, it is possible that peptide presentation by airway DC and pulmonary macrophages within the respiratory tract (discussed in chapters 1 and 5) may also induce direct T cell nonresponsiveness.

The question as to the significance of tolerogenic mechanisms within the respiratory tract remains open and needs to be addressed in future studies. However, there are now recent preliminary data to suggest that a significant amount of intranasally administered peptide is indeed absorbed into the periphery, and gains access to the thymus. In TCR Vβ8.2 PL/J transgenic mice, inhalation of Ac1-9[4Y] appeared to reduce the population of double positive cells in the thymus (Heather Pope & David Wraith, unpublished data). Although the functional significance of this observation remains to be established, it suggests that the intranasal route of peptide administration allows peptide to gain access to the periphery and the thymus.

In order to assess the relative contributions of the respiratory and peripheral lymphoid organs, a direct comparison of intranasally versus i.v. administered peptide will be necessary. However, recent experiments investigating the effect of i.p. injected peptides in the same model revealed very similar results. A single i.p. dose of 100µg Ac1-9 and the 4A and 4Y analogues 1 week prior to priming downregulated the encephalitogenic capacity of T cells with a direct positive correlation between peptide-MHC affinity and the degree of protection from EAE as well as the reduction of in vitro T cell proliferation (George Liu & David Wraith, submitted for publication). Furthermore, the i.p. route of peptide administration in transgenic mice also reduced the percentage of double positive cells among thymocytes (Paul Fairchild, George Liu, and Richard Smith, unpublished data).

If most or all of the N-terminal peptide of MBP exerted its tolerogenic effect in the periphery upon intranasal administration, a very recent publication might shed more light on the possible mechanism. In these studies, Kearney et al monitored the impact of peptide on the fate of a limited number of transferred transgenic T cells specific for OVA 323-339 in normal syngeneic recipients. After s.c. administration of peptide in CFA, these T cells accumulated and proliferated within the paracortex of draining lymph nodes, and were subsequently also detected within follicles. In contrast, after i.v. or i.p. injection of soluble peptide, specific T cells transiently accumulated and proliferated in all lymphoid tissues, but with faster kinetics and without entering follicles. Furthermore, at a later stage, when the majority of T cells had disappeared from the lymph nodes, the few remaining T lymphocytes were more sensitive to restimulation with peptide
after s.c. injection of peptide in CFA (compared to naive transgenic T cells), whereas i.p. or i.v. administration of OVA 323-339 left a small population of hyporesponsive cells that was refractory to further antigenic stimulation both in vivo and in vitro (Kearney et al, 1994).

It is conceivable that peptide inhalation triggered a similar sequence of events. Ac1-9 given by inhalation might have induced early and transient proliferation of encephalitogenic T cells, leaving a small population of these T cells which would then be refractory to further stimulation by subcutaneously injected antigen (Ac1-9 or SCH) in CFA. In this case, rather than anergy alone, the concept of anergy/deletion through activation speculated to be a possible mechanism by which the 4Y peptide operated when given after EAE induction (chapter 6) would also apply to peptide inhalation prior to immunization.

In summary, there is some indirect evidence to suggest that peptide administered intranasally under the conditions applied to all experiments presented in this thesis (chapters 5 and 6) is absorbed into the periphery, where it may induce immunological nonresponsiveness of encephalitogenic T cells. The observation that intranasally or i.p. administered peptide may have an impact on thymocytes raises the question as to what extent these peptides also contribute to central tolerance in immature T lymphocytes. However, there is no evidence against a role for inhibitory mechanisms within the respiratory tract itself.

7.3 ORAL VERSUS INTRANASAL ANTIGEN ADMINISTRATION

GALT and BALT display striking morphological similarities, yet their ultrastructure and cellular composition appear to differ (discussed in chapter 1). With respect to antigen presentation, several studies suggest that intragastric administration of soluble protein does not compromise the function of APC directly. Intestinal DC could bind antigenic fragments derived from fed protein and activate naive T cells in vivo upon transfer (Liu & MacPherson, 1993). Furthermore, macrophages derived from PP presented orally administered antigen to primed T cells in vitro (Richman et al, 1981). To our knowledge, there is no evidence that the immunosuppressive properties shown by Holt and coworkers for airway and lung DC and macrophages (discussed in chapters 1, 5, and 7.1) also apply to intestinal APC. A common feature of both gut and lung DC is a higher turnover rate compared to epidermal Langerhans cells, which subsequently give rise to the DC population within lymphoid organs (Holt et al,
1994; reviewed in Fossum, 1989). The significance of this higher turnover rate of mucosal DC for the induction of mucosal tolerance is not clear. A recent report demonstrating rapid DC recruitment at mucosal sites as a reaction to an inflammatory response (McWilliam et al, 1994), suggests, however, that it might be an adaptation to strong antigenic challenges, and hence important for immune surveillance rather than immunological tolerance.

Both the oral and intranasal routes of antigen administration can lead to the inhibition of DTH-and inflammatory autoimmune reactions characteristic of TH1 types immune responses in rat and mouse models. One might argue that direct intranasal as opposed to small droplet-sized aerosol antigen administration downregulates TH1 responses by peripheral rather than mucosal-associated mechanisms. As pointed out in the previous section, there is now indirect evidence to suggest that the tolerogenic effect of intranasally administered Ac1-9 (and analogues) may to some extent be accounted for by peptide absorption into the periphery. In other reports such as on intranasal administration of Der P1(Hoyne et al, 1993) and retinal antigens (Dick et al, 1993) the question of peripheral versus mucosal mechanisms was not directly addressed. In an early study, however, direct intratracheal instillation of soluble metal salts inhibited specific DTH responses to these salts. Intubation of Evans blue by the same route revealed that the dye penetrated throughout the lung (Parker & Turk, 1978). This finding does not necessarily argue in favour of pulmonary mechanisms to inhibit DTH responses, since antigens may readily be absorbed through the whole of the respiratory tract (discussed in chapter 1). It does suggest, however, that tolerance to TH1 type responses is not confined to intranasal antigen administration by which a high proportion of antigen may bypass pulmonary tissue and be directly absorbed into the periphery.

Both feeding and inhalation of antigen was shown to induce CD8+ and/or CD4+ regulator cells, depending on the experimental model. In one study, aerosolized OVA induced IFNγ-producing CD8+ T cells to abrogate a CD4+-mediated OVA-specific IgE response. This was interpreted as immune deviation from a TH2 response (McMenamin & Holt, 1993). In the Lewis rat model of EAU, the data supported a concept of CD4+ suppressor T cells (Gregerson, Obritsch, & Donoso, 1993) while oral tolerance to EAE in the Lewis rat induced TGF-β producing CD8+ T cells. In the latter model there was also some evidence for immune deviation towards a TH2 response. Immunohistology of brain samples revealed reduced expression of inflammatory lymphokines such as IFNγ and TNFα in MBP-fed rats compared to controls. Furthermore, as mentioned in section 7.1, oral administration of LPS together with MBP had a synergistic protective effect. In this case, brain sections also showed
upregulation of PGE2 and IL-4, implying activation of TH2-specific immune reactions. It is therefore possible that in some models, antigen inhalation might favour immune deviation towards a TH1 response, whereas intragastric administration might selectively downregulate TH1 and also activate TH2 responses under appropriate conditions, such as in the presence of LPS. These findings, however, do not reflect general rules of immune regulation as a consequence of feeding or inhaling antigen. In oral tolerance to OVA, suppression of IgE and IgG titres as well as DTH responses suggested downregulation of all T cell-mediated functions (discussed in chapter 3). The data presented in chapter 3 (table 2) provide no evidence for immune deviation in this model of oral tolerance. Furthermore, as discussed before, the ability to downregulate inflammatory autoimmune reactions by intranasal antigen administration shows that mechanisms other than deviation towards TH1 responses are possible by the respiratory route.

If fed or inhaled antigens exert their inhibitory effect largely in the periphery rather than GALT and BALT, then the respiratory tract is probably a far more effective route of delivering antigens to the periphery than the oral route. This might explain the finding that in contrast to oral administration of Acl-9 and its higher affinity analogues (chapter 4), the intranasally applied peptides induced profound immunological nonresponsiveness (chapter 5). This is not surprising, since secretions and proteases within the gastrointestinal tract presumably hamper effective absorption.

To conclude, it is possible that differences between GALT and BALT favour different modes of immune regulation under some conditions. Presumably, however, these differences are not always the cause of distinct mechanisms for tolerance. Rather, the wide range of phenomena observed in numerous studies on oral and inhalation-induced tolerance might largely depend on the experimental models themselves, and the protocols for antigen administration.

7.4 CONCLUSIONS AND FURTHER STUDIES

The following conclusions can be drawn from the data presented in this thesis.

1. In the Balb/c mouse model of oral tolerance to OVA, feeding 20 mg OVA prior to priming with OVA or the major immunogenic, I-Ad-restricted peptide 323-339 of OVA reduced specific in vitro lymphocyte proliferation in response to OVA or OVA 323-339 by 40-80% compared to PBS-fed controls. Transfer experiments with spleen cells or serum from OVA-fed donors under conditions
previously shown to suppress DTH responses in recipients, could not substitute for direct OVA feeding to achieve this effect. However, oral administration of OVA 323-339 alone did not reduce subsequent T cell proliferation, as might have been expected, if a direct epitope-specific mechanisms such as T cell anergy alone could account for the downregulation of proliferative T cell responses. The inhibition of both IgG1 and IgG2a levels after OVA feeding argues against immune deviation towards TH2 responses as a relevant mechanism in this model.

To understand the mechanism by which oral administration of OVA accounts for the in vitro phenomenon of reduced T cell proliferation, further studies need to address the requirements for distinct OVA epitopes and regulatory cells in more detail. These experiments should involve selective depletion and transfer of CD4+ and CD8+ T cells as well as oral administration of both MHC class II and class I-restricted peptides under various conditions.

2. In contrast to oral tolerance to OVA, there was no evidence for oral tolerance in the H-2u mouse model of EAE when either whole porcine MBP, mouse myelin, or the dominant epitope of MBP Ac1-11, and the higher affinity analogue 4A were fed prior to EAE induction. The time frame for feeding and the effective doses relative to the body weight were comparable with previously applied protocols for oral tolerance in the Lewis rat model of EAE and EAU, showing both T cell-mediated suppression and suppressor-independent mechanisms of nonresponsiveness. Complete abrogation of in vitro T cell proliferation and EAE after oral administration of 3 mg of the 4Y peptide showed, however, that encephalitogenic T cells could be tolerized by peptide feeding, most probably independently of CD8+ T cells. These studies need to be extended to cover a wider range of antigen doses and timing for feeding (preferably pure recombinant murine) MBP, Ac1-9, and both higher affinity analogues 4A and 4Y.

3. Unlike oral administration, a single intranasal 100 μg dose of Ac1-9 (or Ac1-11) and the higher affinity analogues 4A and 4Y inhibited EAE induced with Ac1-9 alone or whole SCH. The ability to induce nonresponsiveness with this I-Au-restricted peptide alone and the positive correlation between peptide-MHC class II affinity and the degree of protection argue against a role for CD8+ T regulator cells. Instead, the data are consistent with a direct epitope-specific mechanism favoured by high avidity TCR-MHC-peptide complexes. Although peptide inhalation also protected against SCH-induced EAE, co-immunization experiments using Ac1-9 and 35-47 of MBP provided no evidence for bystander regulation in this model. However, other data not presented here suggest that immunization with whole myelin (rather than a mixture of peptides) after
inhalation of the dominant epitope Ac1-9 may reveal downregulation of the response to the subdominant epitope 35-47 in a bystander fashion. Clearly, intranasally administered peptides can also modulate the functions of encephalitogenic T cells after the induction of EAE with SCH. Under these conditions, however, the effect of inhaled peptide is not yet predictable, but appears to depend on the activation state of T cells and perhaps also varying precursor frequencies. Significantly, however, data from several experiments suggest that inhalation of the 4Y peptide, and, to some extent also the 4A analogue, may render mice refractory to relapses.

Further studies need to address three major issues. 1) The significance of the respiratory tract for the induction of tolerance after intranasal administration of peptide. Experiments using labelled 4Y peptide are planned to identify relevant APC populations for peptide presentation. T cell stimulation experiments in vitro and transfer studies with DC and macrophages purified from the respiratory tract will address the relevance of these APC for tolerance induction. Additionally, the transgenic model will enable us to locate T cells upon peptide inhalation under various conditions. 2) The potential to induce bystander inhibition to subdominant epitopes upon inhalation of a dominant epitope has to be investigated in more detail. Active bystander regulation mediated by (presumably CD4+) regulator T cells may be revealed in transfer studies with cells from peptide-treated donors. It may be possible to demonstrate lack of bystander help if MBP or Ac1-9 could be modified so as to abrogate processing and presentation and/or T cell recognition of this dominant epitope. 3) On a cellular level, the effect of peptide on the activation state of T cells should be studied. This will be crucial to predict an effective application of peptide after EAE induction, and will also provide information about the mechanism by which peptide acts upon naive T cells (i.e. before EAE induction) at a biochemical level. This issue as well as the questions of epitope specificity and the potential for bystander regulation should be investigated in a chronic-relapsing model of EAE such as in BIOZZI mice which probably provides a more faithful representation of the human autoimmune condition in multiple sclerosis. Here, peptide inhalation between two phases of disease should demonstrate whether previously activated T cells can be prevented from inducing relapses and, furthermore, whether spreading to epitopes other than the dominant initial disease-inducing autoantigenic fragment can be prevented by intranasal administration of the immunodominant peptide alone.

Once detailed knowledge of the mechanism allows us to predict the effect of inhaled antigens on the immune system, this method of antigen-specific immune intervention might prove a convenient and efficient strategy to enforce an
interpretation of those antigens as being innocuous that were erroneously attacked as detrimental.
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