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UNIVERSITY OF ALBERTA

EXPRESSION AND RECOGNITION OF SELF MHC CLASS II MOLECULES

By

© BABITA AGRAWAL

A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

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The UNIVERSITY *of* WESTERN ONTARIO
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March 25, 1993

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Yours sincerely,

Bhagirath Singh

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DEDICATION

To

Amogh, Rakesh

&

My parents

Ravindra Nath Agrawal and Asha Agrawal

WITH LOVE AND HONOR

ABSTRACT

Major histocompatibility complex molecules act as restriction elements for antigen recognition by T lymphocytes. Helper T cells and cytotoxic T cells recognize antigens in the context of MHC class II and class I molecules respectively. MHC class II molecules are highly polymorphic cell surface glycoproteins and are comprised of α and β chains.

We investigated the stimulation of T cell proliferation by synthetic peptides corresponding to the amino-terminal polymorphic domain of α and β chains of self I-A^d molecules. Both primed and unprimed T cells responded to a number of peptides. The response was dependent upon the presentation of I-A^d peptides by syngeneic antigen presenting cells and was blocked by anti MHC class II monoclonal antibodies. Upon further investigations, we found that I-A^d peptides could inhibit the stimulation of antigen specific MHC class II restricted T cell hybridoma due to self presentation of these peptides in the context of I-A^d molecules. The peptide I-Aβ^d 62-78 showed high affinity towards intact self MHC class II molecules and yet was nonstimulatory for syngeneic T cells. Our results further suggest that during ontogeny, developing T cells encounter epitopes of self MHC class II molecules presented by self APCs and become primed *in vivo*. A peptide (residue 62-78) corresponding to an immunodominant region of I-Aβ chain, however represents an epitope which may have induced self tolerance. The tolerance to this specific epitope (I-Aβ 62-78) seemed to be evaded at an older age.

Presentation and recognition of an antigen is dependent upon the level of MHC expression on the cell surface. We have investigated the effect of peptide ligands on the expression of MHC class II I-A^d molecules on a B cell hybridoma, TA3. We found that addition of peptides having high affinity for binding the intact I-A^d molecules increased the surface expression of I-A^d. The effect could be reversed by removing peptide antigen in a time dependent manner. The mechanism for upregulation of I-A^d expression by peptides seemed to involve endosomal compartments in the intracellular pathway. Thus our results suggest that peptide ligands derived from self molecules may be important in regulating cell surface expression of MHC class II molecules and their recognition by T lymphocytes.

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ABBREVIATIONS

ab	Antibody
Ig	Immunoglobulin
TCR	T cell receptor
Ag	Antigen
MHC	Major Histocompatibility Complex
CD	Cluster determinant
cDNA	complementary DNA
V	Variable
D	Diversity
J	Junctional
C	Constant
Ia	I region associated
HLA	Human leukocyte antigen
N-terminal	Amino terminal
C-terminal	Carboxy terminal
APC	Antigen presenting cell
h	Hours
CTL	Cytotoxic T lymphocytes
HA	Hemagglutinin
BFA	Brefeldin A
ER	Endoplasmic reticulum
TAP	Transporter associated protein
LMP	Low molecular mass polypeptide
ATP	Adenosine tri phosphate
CDR	Complementarity determining region
Fab	Fragment antigen binding

IP3	Inositol tri phosphate
DAG	Di acyl glycerol
Ii	Invariant chain
TGN	Trans Golgi network
^0C	degree Celsius
SDS	Sodium dodecyl sulphate
Hb	Hemoglobin
HPLC	High performance liquid chromatography
MAb	Monoclonal antibody
Mls	Mixed lymphocyte stimulatory
SEB	Staphyloccocal Enterotoxin B
IDDM	Insulin dependent Diabetes mellitus
NOD	Non obese diabetic
Asp	Asparatic acid
Ser	Serine
EAE	Experimental allergic encephalomyelitis
MBP	Myelin basic protein
RA	Rheumatoid arthritis
TH	T helper lymphocyte
PPD	Purified protein derivative
Con A	Concanavalin A
FBS/FCS	Fetal bovine/calf serum
BOC	Butyloxycarbonyl group
IL	Interleukin
CFA	Complete freund's adjuvant
LNC	Lymph node cells
HBBSS	Hank's balanced buffered salt solution

GA	Glutaraldehyde
CPM	Counts per minute
HV	Hyper variable
^{3}H Tdr	tritiated thymidine
HEL	Hen's egg lysozyme
FACS	Fluorescence activated cell sorter
MFC	Mean fluorescence channel
Fig.	Figure
Chx	Cycloheximide
Clq	Chloroquine
FITC	Fluorescein iso thio cyanate
Conc.	Concentration

CHAPTER I

INTRODUCTION

The characteristic features of an adaptive immune response are specificity, inducibility and memory. The immune system also has the ability to discriminate between self and nonself. There are two interconnected arms of an adaptive immune response: the humoral and the cellular responses. The humoral response is mediated through antigen-specific receptors called antibodies (ab) or immunoglobulins (Ig) that are on the surface of, or secreted by, B cells. The adaptive cellular response is mediated by antigen specific receptors on the surface of T cells. The antigen specific receptors on both B and T cells are clonally expressed. Antibodies and T cell receptors (TCR) share many structural and genetic features but differ from one another in three very significant ways. Firstly, Ig recognizes intact soluble antigen (ag) whereas the TCR recognizes fragments of antigen. Secondly, the TCR recognizes antigen fragments in an major histocompatibility complex (MHC) restricted manner. Finally, T cells mature in the thymus where selection takes place; no equivalent specific organ for the selection is known in B cell maturation process. The focus of this thesis is on determining how class II molecules and peptide fragments derived from their primary structures induce and regulate T cell mediated immune responses, using murine system as a model. The following section will review the literature on the T cell arm of the immune system.

1. T CELL MEDIATED IMMUNE RESPONSE

The adaptive cellular immune response is carried out by T cells and the specificity of the response is conferred by clonotypically expressed T cell receptors (TCR). T cells with the appropriate specificity contact antigen in the context of major histocompatibility complex molecules via their TCR and are induced to proliferate and initiate effector functions. Clonal expansion in response to an antigen is the basis for a specific attack on the invading pathogen. In the following section, various aspects of T cell mediated responses will be discussed.

a. Structure of TCR and generation of diversity

The TCR is a heterodimer of two disulphide-linked polymorphic glycoprotein chains (1-3). The TCR is coexpressed with a noncovalently associated cluster of monomorphic polypeptides, the CD3 complex (4-5). CD4 and CD8 are nonpolymorphic accessory molecules, that associate with the polymorphic heterodimer of TCR during antigen presentation. Both CD4 and CD8 are involved in signal transduction in T cells upon activation and are not involved in the specificity of antigen recognition. Two subsets of T cells bearing either α/β or γ/δ (6) TCR are found in the periphery. Both antigen specificity and MHC restriction of a T cell are provided by the polymorphic TCR heterodimer (7, 8). Studies involving immunoprecipitation and peptide mapping (9, 10) indicate that the

TCR has regions of constant and variable structure, which are homologous to Ig domains. The complete primary structure of the TCR has been derived from specific cDNA sequences of T cell clones. A tertiary structure of the TCR has been proposed from comparisons of amino acid (aa) sequences of TCR αβ chains and X-ray crystallographic analysis of immunoglobulins (12). The polymorphic glycoprotein chains of the TCR heterodimer are proposed to possess two characteristic Ig domains formed by intrachain disulphide bonds: a variable amino terminal and a constant carboxy terminal domain (13). The TCR αβ chains show similarity to Ig in the size of their putative antigen binding loops and structure of their framework, together with chain connectivity and loop packing. Loops connecting the β strands of the variable domain at the distal amino terminal of the molecule contain a hypervariable region of great diversity. In the proposed model, the hypervariable regions form the antigen binding site and would interact with Ag-MHC complex.

The genes encoding the TCR chains are organized in a manner similar to Ig genes. The variable region of TCR chains are encoded by one each of several Variable (V), Diversity (D only in case of β and δ chains) and joining (J) region genes and the constant (C) region is encoded by a C gene segment. Diversity in the TCR genes is provided by combinatorial joining of gene segments through gene rearrangements and imprecise joining. Additional diversity in TCR is generated by combinatorial association of two polypeptide chains (either α/β or γ/δ) (14). Somatic hypermutation (which is found in Ig

genes), does not contribute to the generation of diversity in TCR genes (14).

b. Structure of MHC molecules and polymorphism

The major histocompatibility complex (MHC) of mammals is a multigene family whose members encode cell surface glycoproteins involved in self/nonself discrimination, selection and activation of T cells and presentation of antigen to T cells. Three families of MHC genes have been identified and are denoted as class I, class II and class III. The class I and class II molecules are typified as transplantation antigens and Ia (I region associated) antigens. These molecules are integral membrane proteins involved in presentation of antigens in cellular interactions in an adaptive cellular immune response. The class III family encodes several complement components. The murine MHC is also known as the H-2 complex (15) and is divided into six regions called K, I, S, D, Qa and Tla. The classical class I molecules ie. transplantation antigens are encoded by K, D and L, whereas the class II molecules are encoded by IA, IE, Ma and Mb region genes. Class I molecules of mice are present on virtually all somatic cells whereas class II molecules are present on a limited number of cells, e.g. B cells, macrophages and dendritic cells. The human MHC is referred to as the HLA complex (16). The human class I MHC molecules are encoded by A, B and C region genes and the class II molecules are encoded by DP, DQ, DR and DM (17) genes. The class I and class II molecules have structural homologies with similar domain structure (18). The class I

polypeptide chain contains three external domains, a transmembrane region and a cytoplasmic domain. The third domain is noncovalently associated with β2m (β2m is not encoded in the MHC). The class II molecules are composed of two noncovalently associated glycoprotein chains, α and β. Each of these chains has two external domains, a transmembrane region and a cytoplasmic domain. Protein and cDNA sequence analysis of alleles of class I molecules demonstrated that the first and second domains are highly polymorphic. Different alleles of class II show high polymorphism in the first external domains (α1, β1). The fundamental mechanism for generating polymorphism in class I has been suggested to be the gene conversion from one class I gene to another in the gene family (19). The mechanism to diversify class II genes is not clear, but has been suggested to occur by mutation, gene recombination and selection (20, 21).

The polymorphic amino terminal α1 and β1 domains of class II molecules and α1, α2 domains of class I molecules appear to be involved in allorecognition and in recognition by ag-specific T cells (22-25). The two classes of MHC molecules have functionally distinct roles. The MHC class I molecules present endogenous ag to CD8 positive cytotoxic T cells and class II molecules present exogenous ag to CD4 positive helper T cells (26). CD4 and CD8 molecules associate with the membrane-proximal invariant regions of class I and class II molecules respectively (27, 28), and are involved in signal transduction during T cell activation (30, 31).

The three dimensional structure of class I molecules has been determined by X-ray crystallography (32). The nonpolymorphic membrane proximal α3 and β2m domains have a tertiary structure similar to Ig domains but are paired by a novel interaction. The polymorphic membrane distal α1 and α2 domains are nearly identical to each other in structure. They do not show similarity to Ig C or V domains and form a platform composed of a single β pleated sheet topped by α-helices with a long groove between the helices. The most polymorphic residues are positioned in or near the antigen binding cleft (33). In the crystals of a class I molecule, the site contained a bound molecule(s) of unknown origin, which was suggested to be peptide antigen(s). The tertiary structure of class II molecules has not been determined but has been modeled by comparing the patterns of conserved and polymorphic residues of a number of class I and class II amino acid sequences (34). Like the class I structure, class II molecules would have a cleft between C-terminal α helices of its polymorphic α1 and β1 domains with the bottom of the cleft formed by N-terminal β strands of each domain. The distribution of polymorphic residues indicates that some of the polymorphic residues probably control interaction with ag whereas others control allele related recognition by T cells.

c. Antigen Processing

The TCR recognizes fragments of an antigen as presented by a histocompatible APC ie. in an MHC-restricted manner. T cells bearing either CD4 or CD8 recognize ag in the context of MHC class II

or class I molecules. The two types of T lymphocytes draw TCR variable region genes from the same pool (35), but the pathway to generate processed antigen ie. peptides are different and lead to two distinct ag processing pathways respectively.

A number of experimental observations suggest that MHC class I molecules present endogenous peptide antigens. Transfection of minigenes encoding short peptides encompassing a CTL epitope sensitized the cells for lysis by appropriate CTL (36). Transfection of a truncated gene lacking the NH2 terminal signal sequence sensitized the cells to lysis by HA (Hemagglutinin) specific CTL (37). This result suggested that HA can be expressed on the cell surface in a form that can be recognized by CTL by a route which is distinct from signal sequence-dependent ER translocation. In a vaccinia virus infected cell, ag processing could be favored by manipulating a gene that resulted in destabilization of the protein (38). This result emphasized the association between degradation of endogenously synthesized antigens in the cytoplasm and recognition by class I-restricted CTL. MHC class I restricted ag presentation can be inhibited by brefeldin A (BFA) (39). It has been found that BFA specifically blocks the translocation of proteins from the ER to the Golgi apparatus. Its effect on class I-restricted antigen presentation supported the idea that endogenous protein antigens processed via cytosolic route associate with newly synthesized class I molecules.

A specific mechanism to generate peptides in the cytoplasm and subsequently transport them to the endoplasmic

reticulum (ER) is required, because class I molecules are cotranslationally inserted into the ER and enter the secretory pathway. Two genes that belong to a family of related transport proteins are encoded in the MHC class II region of mouse (40), rat (41) and human (42). These genes are called TAP-1 and TAP-2 for transporter associated with ag processing. Transfection of TAP-1 cDNA into a TAP-1 mutant cell results in complete restoration of MHC class I surface expression (43) and the ability to be recognized by ag-specific CTL (44). TAP-1 and TAP-2 gene products associate as a heterodimer. It is thought that TAP heterodimer is responsible for transporting fragments of antigens produced in the cytoplasm into the lumen of ER (45).

Genes encoding two of the subunits of a large structure called the low molecular mass polypeptide (LMP) complex also map to class II region of MHC (46). This large cytoplasmic structure is antigenically and biochemically related to proteasomes, large cytoplasmic proteolytic complexes that degrade cytosolic and nuclear proteins. These particles have been implicated in ATP-ubiquitin dependent proteolysis and in the processing of intracellular antigens for cytolytic T cell immune responses (47). Both LMP genes and transporter proteins are polymorphic, and may exhibit differences in function. Polymorphism in LMP genes may result in the production of different sets of peptides in different individuals whereas polymorphism in transporter genes may result in different sets of peptides being imported to the lumen of the ER. The end result could be the presentation of different epitopes of the same ag to the T cell

repertoire in different individuals. Indeed, functional polymorphism in class I ag processing has been suggested (48). In summary, class I restricted ag processing would include production of peptides by the LMP (redefined as a large multifunctional protease) complex, delivery of peptides from the LMP complex to the transporters which reside in ER membranes, and delivery of peptides by the TAP transporter protein to newly synthesized MHC class I molecules in the ER lumen. Another mechanism to provide endogenous peptides has been suggested to be the classical signal-dependent mechanism. The peptides may even be derived from the signal sequence itself (49). Chaperonin like molecules (50) may detain empty class I molecules in the ER to enhance their ability to obtain peptides. The MHC class I molecules bound to the peptide are then transported to the cell surface via the secretory pathway.

Antigens that enter an antigen presenting cell (APC) from the external milieu via the exogenous route are presented by class II molecules. In the case of class II-restricted antigens, antigen processing encompasses the metabolic events that a soluble protein antigen must undergo in or on the APC before it is modified to interact with MHC molecules and recognized by T cells. These events involve uptake of ag by accessory cells, its partial degradation or denaturation and its reexpression on the cell surface. A number of observations form our current understanding of class II-restricted ag processing (51); i) T cells can recognize chemically denatured forms of ag, ii) Small peptide fragments of ag are effective in a T cell response, iii) There is an approximately 1h delay between ag binding

to accessory cells and ag presentation to T cells, iv) The neutralization of the acidic compartment by lysosomotropic agents, such as chloroquin, primaquine and ammonium chloride inhibits antigen presentation by MHC class II molecules (52). The endocytosed ag first enters early endosomes, then late endosomes and finally lysosomes. The concentration of proteolytic enzymes required for the breakdown of endocytosed ag may be achieved towards the late endosomal/lysosomal portion of the endocytic pathway. The morphology and enzyme content of endosomes vary in different stages of cell cycle and differentiation (53). It is possible that different cell types that express the same class II molecules present different peptides generated from the same antigen at various stages. The MHC class II molecules traverse the endocytic pathway *en route* to the surface following their biosynthesis (54) and acquire exogenous antigens from endosomal compartments.

Apart from exogenous antigens, endogenous peptides can apparently bind to MHC class II $\alpha\beta$ dimers during their assembly in the ER. The association of invariant (Ii or γ) chain normally prevents binding of peptides (55). However, the possibility that the γ chain can also bind to class II molecules after the binding of peptides in the ER, can not be excluded. When ag is processed or modified in the cytosol or the ER (56), some of the modified ag may be degraded in the endocytic route, or the peptides may be directly delivered from the cytosol to lysosomes resulting in the presentation of endogenous ag by class II molecules. The latter pathway may be distinct from that taken by endogenous peptides presented by class I because

chloroquin can inhibit the presentation of endogenous ag by class II, but not by class I molecules. It is further suggested that the pathway taken by endogenous peptides to class II molecules must be similar to that taken by exogenous ag, because exogenous peptides can compete for presentation of endogenous ag by class II molecules (56).

d. Interaction of TCR, MHC and antigen

The molecular nature of the interaction between the T cell receptor (TCR) heterodimer and its ag/MHC ligand has been speculated by the characterization of the primary structure of TCR and by the three dimensional structure of class I and class II MHC molecules. Distinct portions of the peptide antigen must bind to an MHC molecule (agretope) and also interact with the TCR (epitope). Using synthetic peptides, both agretopic and epitopic sites have been delineated in a number of specific antigen systems (57, 58). A recent study has also delineated the TCR contact site on a peptide antigen by using transgenic mice (59). Direct binding of peptides with MHC molecules has been demonstrated with purified MHC molecules (60) and intact cells bearing the relevant MHC molecule on their surface (61). Class I molecules appear to bind 9 amino acid peptides with highest affinity whereas class II molecules can bind to peptides of 10 to 20 amino acid residues (62). The kinetics of association and dissociation of ag/MHC complexes has also been reported. For a defined ag-MHC class II system, two types of complexes were found to be formed. One complex showed slow apparent association and dissociation kinetics and the second complex had a hundred fold

higher association and dissociation rates. The short lived complex might be an intermediate in the formation of the long lived complex and exhibited peptide-MHC specificity whereas the long lived complex might be recognized by specific T cells (63).

The most recent model for binding of the TCR to the ag-MHC complex (64, 65) suggests that both chains of the TCR have the capacity to interact with residues of α1 and α2 domains of the class I molecule via CDR1 and CDR2 loops and with the ag via their CDR3 loops. The binding affinity of the TCR to its ligands has also been quantified by determining the amount of soluble MHC class II antigen loaded with a relevant peptide that was needed to block the binding of an anti-TCR Fab of known affinity to cell bound TCR (66). In another study (67) T cell responses were blocked with soluble forms of TCR. These two studies suggested that the affinity of T cell-ligand interaction is low and is in the order of the lowest known antibody reaction for binding. In view of the low affinities and the requirement of a small number of peptide-MHC complexes on presenting cells that can activate T cells (68), it seems more likely that initial event in T cell recognition could be the cell-cell contact initiated by adhesion molecules. The initial contact by nonantigen-specific adhesion molecules may be followed by congregation of correct MHC/peptide at the interface. This would promote high local density of TCR-CD3 complexes, leading to T cell activation.

e. Effector functions of T lymphocytes

TCR interaction with its ligand is followed by receptor clustering which activates receptor associated tyrosine kinase activity, with a consequential activation of Phospholipase C (PLC), which induces cleavage of phosphoinositol (PI) into inositol-triphosphate (IP3) and diacyl-glycerol (DAG). These second messengers trigger many downstream signalling events (69). In addition to the specific ligand binding receptors and their associated chains, other cell surface molecules ie. CD4 and CD8 also contribute to transmembrane signalling in T cells (69). Lymphocytes require two signals in order to proliferate, differentiate and carry out effector functions. Antigen specific signals are delivered via the antigen receptor and costimulatory signals are delivered from other cells, ie. APCs or by other T cells. T cell activation also leads to *de novo* expression of a number of cell surface molecules which are involved in lymphocyte homing to sites of infection or autoantigen expression. The function of T cells is the specific (69) identification and alteration of target cells. Some of the effector functions of T cells are brought about by soluble secreted products called cytokines that act on cell surface receptors. The cytokines are not ag specific but their directed release may lead to the specificity of T cell effector function. Besides the soluble secretory products, physical contact between ag-specific T cells and target cells are required for three major effector functions of T cells, ie. B cell activation to ab production, macrophage activation and cell mediated cytotoxicity. T cells have been found to deliver essential contact dependent signals. These contact dependent signals would impart specificity on the target cell activated. Thus T cells seem to have two distinct forms of effector molecules, soluble and

membrane bound, and both act upon specific cell surface receptors (69).

2. EXPRESSION OF MHC MOLECULES

The level of MHC class I and class II antigens on a cell is not a fixed characteristic and is subject to wide variations. The resting level of expression of MHC molecules varies widely in different cell types, and can be upregulated, by exposing the cells to various cytokines released during inflammatory responses, and also in response to a number of other non-specific stimuli.

a. Intracellular transport

The class I and class II MHC molecules differ in biosynthetic assembly and intracellular trafficking, which contributes to their acquisition of antigenic peptides from different sources. The two subunits of class I molecules are targeted to the ER by classical amino terminal sequences that are cleaved off once the molecules are cotranslationally translocated into the ER. An ER retained chaperonin-like molecule may assist in assembling β2m and the α chain(70, 71). After leaving the ER, class I molecules traffic through the Golgi apparatus and arrive at the cell surface with an average half time of 30 minutes (72).

The α and β chains of class II are cotranslationally translocated into the ER and have amino terminal signal sequences which are cleaved. The two subunits of class II molecules are

associated with a third subunit, called the invariant chain (Ii or γ) in the ER. The Ii chain has an internal signal sequence. The Ii chains form a trimer and acquire three sets of α and β chains in the ER. The class II molecules traffic through the Golgi apparatus at a rate similar to that of class I molecules, but are still accompanied by invariant chains. Class II molecules have a half time of 2-4 hours for surface expression and a delay for class II molecules occurs between the Golgi and plasma membrane. It has been suggested that the class II molecules traverse the endocytic route on their way to the cell surface from the trans Golgi network (TGN) (72). In the endocytic vesicles, the αβ complex dissociatesd from invariant chain prior to expression on the cell surface.

b. Role of invariant chain for transport

MHC class II molecules are noncovalently associated with Ii chain in the ER but on the cell surface, the majority of class II and Ii do not appear to be associated. A number of hypotheses have been proposed to account for the function of the Ii chain. The first hypothesis is that Ii is required for cell surface expression of class II molecules, this could not be supported, as in transfection experiments, class II could be fully expressed on the cell surface in the absence of Ii (73). The Ii chain has also been proposed to function in ag processing and presentation by blocking or modifying the ag binding groove (74), thereby protecting class II from association with endogenous peptides in the ER or Golgi. The Ii chain may contain a signal to target the Ii/class II complex from the TGN into endosomes

(75). Finally, Ii may keep class II in a conformation with low affinity for peptides. In the endosomes Ii is removed and a high affinity $\alpha\beta$ conformation, more receptive to peptides is acquired (76). In contrast to class II molecules, class I molecules have not been found to be associated with a nonpolymorphic third chain, rather, for efficient expression of class I molecules, association of α chain with β2m appears to be all that is necessary. However, release of class I by the ER retained p88 chaperone molecule might regulate the export of class I molecule from the ER to the Golgi and on the cell surface (70, 71).

c. Stability and efficiency of transport of MHC heterodimer: influence of occupancy of ag binding groove

The observation that electron dense material is associated with the ag binding cleft of HLA class I molecules and that peptide treated target cells can be recognized by $CD8^+$ T cells, prompted the study of the role of processed ag in the assembly of MHC class I molecules. A causal link between peptide-MHC association and assembly and transport of nascent MHC class I molecules was suggested by the experiments using a mutant cell line RMA-S (77). Exposure of specific peptide to these cells resulted in an enhanced expression of the specific MHC class I molecules at the cell surface, enhanced association of nascent MHC class I heavy chain with endogenous β2m and an increase in the more mature complex carbohydrate form of the molecule. These observations were

followed by the report of peptide-induced upregulation of mouse MHC class I molecules (L^d) in normal cells (78). Furthermore, the mutant cell line RMA-S showed high class I expression at low temperatures. The MHC class I molecules were labile at 37^0C, but could be stabilized by exposing the cell to specific peptides (79). It has thus been suggested that peptide binding is not required for folding, assembly and transport but rather peptides play a role in stabilizing certain MHC class I conformations. In summary, peptides may serve not to only maintain certain preferred conformations but may also aid in assembly and intracellular transport of class I molecules.

A parallel link between ag-processing and MHC class II structure has been investigated in a human lymphoblastoid cell line. This cell line was deficient in presentation of protein antigens to $CD4^+$ T cells but expressed normal levels of class II molecules (80). These processing-deficient cells could present preprocessed peptide antigens to T cells. It was further suggested that MHC class II molecules on the surface of this processing-deficient cell line were unstable because they were empty ie. they lacked peptides in their ag binding groove. Using purified class II molecules (81), it has been shown that the stable binding of peptide is necessary for class II $\alpha\beta$ dimers to enter a compact state that resists dissociation in 1-2% SDS at room temperature. It was found that peptides did not bind to MHC class II molecules before transit through the medial-Golgi. Instead, it seemed to be a chloroquin sensitive process that only occurred in the molecules free of intact invariant chain (82). Addition of antigen

augmented the generation of stable class II dimers and increased overall surface class II molecule expression in the above study. In contrast, a substantial number of apparently empty class II molecules have been reported to reach and reside on the cell surface (83).

Thus, in a related but distinct manner, peptides seem to have a role in transport and stability of both class I and class II molecules.

3. PROCESSING AND PRESENTATION OF SELF ANTIGENS

Discrimination of self from non-self is one of the cornerstones of the immune system. The ag presenting cells do not seem to distinguish between a self or a non-self protein. In addition, the presentation of self antigens by the APC seems to have an impact on the development of the T cell repertoire. The processing and presentation of self proteins will be discussed in the following section.

a. Functional self peptide/MHC complexes in vivo and in vitro

Direct binding to purified Ia (60) and competitive inhibition of T cell stimulation by self peptides (84) provided the first evidence that self peptides can bind to Ia molecules, although many self peptides do not bind to Ia. Direct evidence for the processing and presentation of self proteins has come from a number of studies using

functional assays. It has been shown using IgG2a (85), Hb (86) or HLA3-DR3 (87) specific T cell clones/hybridomas that APCs, directly after removal from the individual, can stimulate specific T cell clones without the addition of exogenous antigens. The *in vivo* presentation of self ag seems to be a widespread phenomenon with functional self peptides/Ia complexes being found on the surface of tissue APCs in most major organs of the body. Thus in a non-disease state, self proteins are processed constitutively and can be presented in a fashion similar to that in which foreign antigens are presented.

b. Extraction of naturally processed peptides

The direct association of peptides with MHC molecules was first visualized by X-ray crystallographic studies of MHC class I molecules. Development of acid extraction methods, ie. utilization of the fact that most peptides are soluble under acidic conditions, provided direct access to MHC bound peptides. The peptides bound to MHC can be acid extracted, separated by HPLC techniques and examined for their antigenicity. Fractions containing naturally processed antigens are further characterized by sequencing or by comparing their elution behavior with those of synthetic peptides. Capillary electrophoresis-electron spray ionization-tandem mass spectrometry is a much more sensitive approach which has been used to analyze the peptides bound to MHC molecules (88). The peptides bound to human class I molecules have been analyzed using narrowbone HPLC techniques (88, 89). Most of the peptides bound by class I molecules were 9 amino acids long and seem to have a distinc

structural motif for a specific HLA molecule. The majority of the eluted peptide sequences were derived from abundant cytosolic or nuclear proteins. Peptides, eluted from class I molecules of virally infected murine target cells, included the endogenously synthesized viral peptides (90, 91), which are presented in the context of MHC class I molecules. Furthermore, it has been observed that MHC molecules are themselves substantially involved in defining the intracellular peptide pool (92), since normal spleen cells identical at all genes but MHC class I, express different patterns of peptides derived from cellular proteins.

Peptides bound to murine (93, 94) and human (95) MHC class II molecules have also been analyzed. Predominant naturally processed peptides bound to class II molecules are derived from self proteins, are heterogeneous in size and have variable (ragged) carboxy and amino termini, although peptides bound to a specific MHC class II molecule show a putative binding motif. From a murine class II I-A^d molecule (94), 650-2000 different peptides were found to be associated. Analysis of the sequences of some of these peptides suggested that these peptides were derived from secretory or integral membrane proteins that are synthesized by the APC itself.

4. THYMIC SELECTION

Multipotential hemopoietic precursors from the fetal liver and thereafter from the bone marrow colonize the thymus at about day 11 of murine fetal life and continue to migrate into the thymus

throughout adult life. Upon arrival in the thymus, double-negative T cell precursors undergo extensive gene rearrangement, phenotypic alteration and biochemical modification to yield the population of thymocytes that undergoes intrathymic selection. There are two categories of intrathymic selection: positive and negative, which will be discussed in the following sections.

a. Positive Selection

T cells recognize fragments of protein antigens in the context of MHC molecules when the MHC molecule is of the same genotype as the T cells. The self MHC bias of the TCR is not encoded in the TCR genes, but is acquired during T cell maturation in the thymus. The earliest demonstration of positive selection came from experiments involving radiation bone marrow chimeras, in which T cell depleted bone marrow cells of (P1 X P2)F1 progeny were injected into irradiated host of either parental strain. The F1 T cells acquired the MHC restriction of the host parental strain, not that of the F1 (96) or the other parental strain. Subsequent experiments using thymectomized mice indicated that the MHC type expressed by the thymus determines the restriction of the T cells (97), suggesting the role of the thymus in positive selection. Experiments using chimeras have the disadvantage of residual macrophages or lymphocytes in the irradiated host. This has been overcome by using transgenic mice and clonotypic antibodies for TCRs as tools.

The compelling evidence for the role of MHC molecules in positive selection came from the demonstration that new born mice treated chronically with anti-MHC class II (98) or class I (99) specific monoclonal antibodies (MAb) failed to develop $CD4^+$ and $CD8^+$ T lymphocytes respectively. In addition, mice lacking the expression of class I (100) or class II (101) have greatly reduced levels of $CD8^+$ or $CD4^+$ T cells respectively.

The fact that interaction of MHC and the TCR is required for positive selection, became evident from a study using mice transgenic for a rearranged TCR β chain lacking the Vβ region. These mice lacked $\alpha\beta^+$ thymocytes and $CD4^+$ or $CD8^+$ thymocytes (102). In normal Mls-1^b mice, the frequency of $V\beta6^+$ T cells is higher in mice expressing I-E of k or d haplotype than the mice lacking I-E (103). Similarly, the frequency of $V\beta17^+$ T cells is higher in mice of the H-2^q haplotype than that of mice of the H-2^b haplotype (104). Mice transgenic for a TCR specific for a cytochrome c (cyt c) and I-E^k, have elevated $CD4^+$ T cells only when the mice express I-E^k (105). Mice of the H-2^b haplotype which are transgenic for the same TCR have increased levels of double positive thymocytes but have few $CD4^+$ T cells, further supporting the phenomenon of positive selection in T cell development (106). In another study, mature $CD8^+$ T cells were found to develop in female mice transgenic for a TCR specific for male (H-Y) antigen and D^b only if the mice express D^b molecules (107).

The identity of cells that imprint H-2 restriction in the thymus has been a debatable question. Radio-resistant thymic

epithelial cells in the cortical region have been suggested to provide H-2 restriction (108). Using several strains of transgenic mice that express class II MHC molecules I-E in specific regions of the thymus, it has been directly demonstrated that positive selection of T cells is mediated by class II-bearing stromal cells in the thymic cortex (109).

b. Negative Selection

A central tenet of the immune system is the lack of immune responsiveness to self components. Clonal deletion appears as a major mechanism of T cell tolerance. First direct demonstration of clonal deletion was the observation at self reactive T cell clones were absent in mice expressing the corresponding superantigen. Vβ17a$^+$ T cells, which recognize I-E, were absent in the periphery of I-E expressing mice strains (110). Examination of TCR expression in the thymuses of these animals showed that clonal deletion occured during thymocyte development. There was significant expression of Vβ17a$^+$ cells in immature thymocytes, and Vβ17a$^+$ cells were virtually absent in the mature medullary population of thymocytes, thus suggesting that the thymus was the site of negative selection. Mice expressing Mls-1^a delete T cells bearing Vβ8.1, Vβ6 and Vβ9 (111). Vβ3$^+$ T cells were absent from Mls-2^a or Mls-3^a expressing mice (112). Clonal deletion in the thymus has also been induced experimentally *in vivo* and *in vitro*. *In vivo* injection of Mls-1^a (113) or SEB (114) into neonatal mice or addition of bacterial enterotoxin to fetal thymic organ cultures (115) results in the efficient elimination

of the mature thymocytes expressing the Vβ reactive with the antigen.

Clonal deletion has also been observed in the thymuses of the TCR αβ transgenic mice that express the antigen and relevant MHC type (116). It was demonstrated that deletion of autospecific cells occured at the $CD4^+8^+$ double positive nonmature-thymocyte stage. Other evidence also indicate that deletion acts on double positive thymocytes. First, both $CD4^+$ and $CD8^+$ T cells bearing Vβ17 are eliminated in I-E positive mice even though only $CD4^+$ T cells are reactive to I-E (110). Second, treatment of developing thymuses with antibodies to CD4 or I-E allows the development of $CD8^+$, Vβ17a bearing T cells in I-E expressing mice (117). Finally, neonatal treatment of Mls-1^a expressing mice with anti-CD4 resulted in the appearance of $CD8^+$, $V\beta6^+$ mature T cells (113).

The identity of the cells that are capable of inducing clonal deletion is still not completely solved. The corticomedullary junction seems to be the site of deletion in the thymus, since $V\beta6^+$ T cells are found in the cortex but not in the medulla of Mls-1^a mice (118).

c. The mechanism of positive and negative selection

The affinity model of thymic selection (119) suggests that an immature T cell bearing a TCR with strong affinity for self antigen will be deleted when it encounters that self antigen. Positive

selection is the rescue from cell death of those immature T cells which have some affinity for self MHC molecules.

The mechanism of negative selection appears to be the induction of programmed cell death, ie. apoptosis. Several studies show that signalling through the TCR of immature thymocytes (expressing low levels of CD3) by either exposure to anti-CD3 MAb (120) or antigen (121, 122) can induce the immature thymocytes to die by apoptosis. Another model suggests that MHC molecules on selecting cells in the thymus are different from those in the peripheral tissue. Novel MHC molecules (123) have been observed in the medulla of the thymus. Alternatively it has been suggested that the self peptides bound to the MHC molecules in the thymus are novel. There is some evidence to suggest that thymic epithelial cells differ in processing and presentation of antigen. The radiation bone marrow chimeras $(K^b \times K^{bm3})F1 \rightarrow K^{bm3}$, in which K^{bm3} differs from K^b only by a few residues in the ag binding groove, were unable to positively select for T cells able to respond to ovalbumin and K^b (124), in spite of the presence of K^b expressing APCs in the periphery.

CD4 and CD8 molecules are critically important in both positive and negative selection. It has been suggested (125) that the two coreceptors transduce distinctive signals during positive selection in double positive thymocytes. The two signals might share the property of inducing T cell maturation, but in the immature thymocytes, CD4 signals may program the cell to repress CD8 expression, and *vice versa* It has been shown that by decreasing the

contribution of CD8 (126), the receptor affinity required for both positive and negative selection is increased, ie. T cells whose receptors have relatively high affinities for self MHC may survive negative selection. Thus, besides the affinity of TCR towards its ligands, interaction and signalling through the coreceptor play an important role in T cell selection events.

5. TOLERANCE

Tolerance is the absence of immune reactivity to self molecules. Negative selection of developing T cells, reactive to the self antigens is the major mechanism of tolerance induction for the self antigens expressed in the thymus. Tolerance to self antigens, that are not constitutively expressed in the thymus, takes place in the extrathymic milieu. A number of studies suggest extrathymic deletional and nondeletional mechanisms for the establishment of tolerance to extrathymic self antigens.

a. Extrathymic deletion

Several experimental observations have suggested that clonal deletion of T cells may occur in the periphery. No distinct site or cell type has been proposed to be specific in inducing clonal deletion extrathymically. Mls-1^a mice, thymectomized and treated with anti-class II MAb neonatally, gradually loose V$\beta6^+$ T cells from the periphery (127). Similarly, injection of Mls-1^a spleen cells into a thymectomized Mls-1^b recipients causes an initial expansion of V$\beta6^+$

T cells (128). Similar results have been observed in male nude mice injected with cells from mice transgenic for H-Y antigen and D^b (129). The initial encounter of mature T cells to the superantigen in the periphery may be followed by their deletion or anergy.

b. Anergy

A primary alternative for the nondeletional mechanism of tolerance is clonal inactivation. Inactivation of T cells has been hypothesized to occur by exposure of T cells to antigen plus MHC molecules in the absence of a second, costimulatory signal. Many examples of T cell anergy have been elucidated *in vitro* (130), including inactivation of T cell clones via antigen presented by human T cell clones, chemically modified APC and purified MHC class II molecules in planar membranes. The *in vitro* observations of clonal anergy have been extended as a mechanism of peripheral tolerance in recent reports where the anatomical expression of self antigens was experimentally manipulated (131). Intravenous injection of Mls-1^a bearing spleen cells into adult Mls-1^b mice does not cause Vβ6 bearing T cells to disappear, but does render them anergic. Similarly, transgenic mice with defined expression of MHC proteins have provided novel tools to study peripheral tolerance *in vivo*. The MHC class II I-A and I-E and class I H-$2K^b$ molecules were expressed specifically on pancreatic islet or acinar cells, and served as models for self antigens (132). In these mice, transgenic proteins were not detected in the thymus or lymphoid tissue. Yet, mice were tolerant to the pancreatic MHC products *in vivo*. This tolerance was not induced

by clonal deletion. The use of MAb specific for I-E reactive T cells further indicated that clonal anergy might be an important mechanism of tolerance to extrathymic proteins. T cell clonal anergy has also been suggested to be one of the mechanisms for induction of tolerance to intrathymic self antigens (133) during T cell maturation in the thymus.

c. Clonal Suppression

Clonal suppression mediated by T cells has been defined in two basic circumstances. First, unresponsiveness to an ag in the presence of T cells can be restored by removing T cells. Second, unresponsiveness to an ag can be induced by specific forms of presentation e.g. route of administration, dose of ag, coupling to self ags or chemical modification of ag (134). The molecular mechanisms to explain suppression are still controversial. Recently, a rather simple model has been proposed (135) based on functional subsets of $CD4^+$ and $CD8^+$ T cells and on their pattern of lymphokines secreted. The suppressor cells are the cells proposed to be different from other functional subsets, have specificities for idiotypes of other TCR, and suppressor factors may be shed TCR molecules and secreted cytokines. The previous model of suppression proposed a complex cascade of suppressor inducer, suppressor transducer and suppressor effector T cells and soluble suppressor factors, with differing restrictions and specificities (136). Both of these models suggest that the suppressor cells have antiidiotypic specificity. It has also been suggested that presentation of peptide fragments of the TCR by T cells

to suppressor cells may lead to down regulation of the presenting T cells (137). The peptide fragments of TCR could be MHC class I or class II restricted. In summary, the suppressor cells may share common mechanisms for antigen recognition with conventional T cells and may not represent a separate lineage. The nature of the presenting cell may be an important factor in determining the functional phenotype of the responding T cell. In response to recognition of idiotypes presented by T cells, the lymphokines produced may be inhibitory to the presenting T cell, resulting in suppression.

6. AUTOIMMUNITY

Self proteins are continuously processed and presented as peptides in association with self MHC molecules for interaction with T cells (discussed in a previous section). T cells reactive to these self ligands are made tolerant by imposition of clonal deletion, inactivation or suppression during ontogeny. In spite of these mechanisms to ensure self tolerance, T cell reactivity to many self peptides in the context of MHC class I or class II molecules has been observed in studies with transgenic mice carrying genes for soluble H-2^k or self class I (138) or self class II (139) where mice were not tolerant to the introduced molecules (as self). The most intriguing demonstration of self reactivity came from a study using normal mice (140), where CTLs specific for a peptide derived from a ubiquitous autologous protein (hemoglobin) were found to be present. Peripheral T cells responding to peptides of self molecules such as

class I (141), class II molecules (chapter II) and TCR molecules (142) have also been observed. The presence of self antigen reactive T cells in the periphery does not manifest the pathological conditions. The activation and recruitment of these cells may be necessary for the development of an autoimmune disorder. Most autoimmune diseases are associated with MHC and non-MHC gene products predisposing the individual susceptible to the disease. However, not all the susceptible individuals develop the pathological condition. An initial viral, bacterial or parasitic infection (143) can either i) cause tissue damage resulting in release of sequestered or cryptic self ag, and the production of local inflammatory cytokines resulting in lymphokine release and subsequent ectopic expression of MHC class I or class II molecules, ii) induce the activation of T cells which are cross reactive to a self epitope or iii) may break tolerance in anergic but potentially autoreactive T cells by activation of alternative reaction pathways (144). In conclusion, autoimmunity is the result of the sum of genetic and environmental factors that override normal mechanisms of self tolerance.

MHC class II associated autoimmunity

Certain MHC class II molecules are found to be associated with susceptibility to autoimmune diseases (145). Insulin dependent Diabetes melitus (IDDM) has been found to be linked with one or more disease susceptibility genes in the MHC class II loci. The β chain of MHC class II molecules in both susceptible humans and non obese diabetic (NOD) mice has a unique β1 domain with several

substitutions including the substitution of Asp at position 57 by Ser (145). Position 57 is located on the α helix of the ag binding groove, and may be important in binding the autoantigen for presentation or the selection of autoreactive cells.

Experimental allergic encephalomyelitis (EAE) is an autoimmune disease mediated by autoreactive T cells specific for MBP and has been linked to MHC class II molecules (146). Inflammatory lesions in the perivascular areas of the CNS contain B cells, T cells as well as class II bearing cells. The T cell repertoire reactive to MBP is very restricted. The MHC molecules play a major role in the immune response to MBP and may elicit disease susceptibility by influencing the selection of MBP reactive T cells.

Rheumatoid Arthritis (RA) in humans has been found to be associated with susceptibility genes in the HLA region (145). Residues 70 and 71 of β chain may be important for the association of the DR4-non Dw10, DR1 and DRw10 haplotypes with RA.

The correlation between class II epitopes and disease susceptibility indicates that for RA, EAE and IDDM, MHC genes contribute directly to disease susceptibility. Disease susceptibility class II alleles may create an ag binding site that is effective in presenting self peptide from synovium, MBP or the pancreatic beta cells to peptide specific T cell clones. Alternatively, they may be defective in presenting a self peptide to T suppressor cells or they may be defective so that the autoreactive T cells are not deleted.

Various forms of immunotherapy can be applied to prevent the onset or progression of MHC class II associated autoimmune diseases. Antibodies to class II can prevent or reverse disease. Protective peptides may be introduced into the susceptible individual which may bind to class II molecules and create epitopes recognized by T suppressor T cell receptors. The levels of class II molecules may be manipulated which may influence disease onset. Clonal deletion or anergy of autoreactive T cells can be induced by using peptides of the putative self antigen. The pathogenic T cells can be deleted using MAb to the autoreactive TCR. In addition, other susceptibility genes and possible environmental factors must be identified in order to detect the susceptible individuals.

PROJECT AND RATIONALE

An enormous amount of information about T cell mediated immune responses has been generated. One general rule which seems to be governing T cell mediated responses is the corecognition of antigenic fragments and Major Histocompatibility Complex molecules by T cells. The structure of MHC class I molecules has been elucidated and the direct association of antigenic peptide with MHC molecules has been visualized. The identity of ligands recognized by T cells has been analyzed in a number of studies involving antigen specific T cell responses. Some of the major but unanswered questions concerning the identity of ligands for T cell recognition are as follows:

1. The phenomenon of autologous or syngeneic MLR has been recognized in a number of *in vitro* experiments, where the T cells are believed to recognize the combination of self peptide and self MHC molecules. The identity of peptides involved in T cell interaction in a syngeneic MLR is unknown.

2. Immature thymocytes are programmed to die unless selected positively by interacting with sufficient affinity with major histocompatibility complex molecules expressed on thymic epithelial cells. It has been previously suggested (123) that tissue specific self peptides or eronous self peptides are involved in positive selection. This idea has been further strengthened by experiments (124) which suggest that variation in the residues which affect the interior but not the exterior of the antigen binding groove, affect the positive selection of T cells. This implies that peptides associated with MHC molecules on the thymic epithelium have a critical role in selecting the T cell repertoire. The identity of peptides involved is unclear.

In the vast array of self and nonself peptides, which could serve as likely epitopes in the two phenomena described above, we speculated that the peptide fragments of MHC molecules may play a role as potential ligands. The reason being that in both these cases, MHC molecules seem to be present as abundant self molecules. Among the two classes of MHC molecules, we decided to investigate MHC class II molecules for the following reasons.

1. After biosynthesis, MHC class II heterodimeric (or trimeric) complexes, traffic from the secretory pathway to endocytic compartment(s) (72). The endosomal environment is likely to lead to

fragmentation of some of the α, β and γ (or Ii) chains of MHC class II molecules producing the peptides which may then be expressed in the context of MHC class II molecules on the cell surface, generating as an epitope for T cell recognition.

2. MHC class I and II molecules are present on the thymic epithelial cells. It is possible that fragments of MHC class II molecules are presented in the context of intact MHC class II molecules on the surface of these cells and influence the selection of maturing thymocytes.

3. Studying one class of MHC molecules narrowed the scope of our investigations.

The two chains of MHC class II molecules, ie. α and β, are comprised of highly polymorphic amino terminal α1, β1 domains and highly conserved α2, β2 domains, transmembrane domains and cytoplasmic domains. Polymorphism at the hypervariable regions of the amino terminal α1 and β1 domains dictates the MHC restriction. Fragments corresponding to the hypervariable regions of α and β chains would be expected to generate specific ligands in an MHC-restricted manner in individuals with different MHC haplotypes in contrast to the fragments corresponding to the conserved region. Therefore, we investigated the fragments of MHC class II α and β chains, corresponding to the hypervariable regions. Synthetic peptides representing the hypervariable regions of amino terminus of α and β chains of MHC class II were used as tools in our studies.

Our first objective was to determine whether T cells from mice could be stimulated to proliferate in response to peptides of

syngeneic MHC class II molecules. We further examined the affinity of MHC class II peptides towards the antigen binding groove of syngeneic MHC class II molecules present on the surface of syngeneic APCs. (Chapter II)

Our second objective was to determine the ontogeny of T cells reactive to the peptide fragments of self MHC class II molecules. The ontogeny studies would lead us to examine the role of MHC class II peptides in positive and negative selection of the T cell repertoire. (Chapter III)

Our final objective was to determine the role of MHC class II molecules in regulating an immune response, since they may be produced *in vivo* and presented in the context of MHC class II molecules. T cell recognition of an antigen is dependent upon the level of MHC expression on the cell surface. We have explored the effect of MHC class II peptides with high affinity for the antigen binding groove of intact MHC class II molecules, on the cell surface expression of MHC class II molecules (Chapter IV). We have also attempted to examine the mechanism(s) by which peptides regulate the surface expression of MHC class II molecules. (Chapter V)

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CHAPTER II

T CELLS EXIST IN SYNGENEIC MICE WHICH RECOGNIZE PEPTIDE SEQUENCES OF SELF MHC CLASS II MOLECULES

INTRODUCTION

Major Histocompatibility Complex (MHC) antigens are polymorphic cell surface molecules with critical roles in self/nonself discrimination by the immune system (1) and in the presentation of antigens to T lymphocytes (2). T helper cells recognize antigens in association with MHC class II molecules whereas cytotoxic T cells recognize antigens in association with MHC class I molecules (3, 4).

In mice, MHC class II molecules are encoded by I region genes (I-A and I-E subregions) and hence also called as I region associated (Ia) molecules (5). I-A molecules encoded by the I-A subregion are highly polymorphic cell surface glycoproteins comprised of α and β chains (6). Both α and β chains are integral cell surface proteins containing membrane distal polymorphic amino-terminal (α_1, β_1) domains and less polymorphic membrane proximal (α_2, β_2) domains followed by transmembrane region and a cytoplasmic tail (7, 8). The polymorphic α_1 and β_1 domains appear to be involved in recognition by allogeneic T cells, allo antibodies and

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antigen-specific T cells by a number of studies involving exon shuffling (9), naturally occurring I-Aβ bm12 mutation (10, 11), site-directed mutagenesis (12) and transfection studies (13). I-A molecules can selectively bind the peptides resulting from the intracellular degradation of self (14-16) as well as non self (17, 18) proteins.

Immunological recognition of self peptide in context of self MHC molecule by T lymphocytes is prevented by imposition of self tolerance, which is established either by clonal deletion during thymic differentiation (19-21) or clonal inactivation (anergy) (22-26) of self reactive T cell clones. Although the mechanism of self tolerance is essential for the maintenance of integrity of self, contradictions to this general rule have been found in studies with transgenic mice carrying genes for soluble H-2^K or self class I and class II antigens (27, 28) where mice were not tolerant to the introduced molecule (as self) and with normal mice where cytotoxic T cell response was found against peptides derived from various self proteins (29). One of the reasons for this failure of self tolerance is attributed to the presentation of self peptides in insufficient density (30, 31).

MHC class II molecules are important and interesting self molecules because they act as restriction elements for antigen recognition by TH lymphocytes and because of their high immunogenicity in allogeneic animals. In both syngeneic and allogeneic MLR, T cells are presumed to recognize the combination of self/foreign peptides and MHC molecules. In the present study the synthetic peptide approach has been used to analyze the

immunogenicity/tolerance of I-A molecules in syngeneic as well as in allogeneic mice. We have used synthetic peptides of I-A^d (both α and β chains) that represent the most polymorphic areas on the I-A molecule. We have analyzed the immunogenicity of these I-A^d peptide fragments in syngeneic and allogeneic mice. Most of the I-A^d peptides were found to be immunogenic in allogeneic mice as expected (32). Our results further indicate that both primed and unprimed syngeneic T cells respond to selected I-A peptides in presence of syngeneic APCs, this response could be inhibited by monoclonal antibody to I-A^d. These I-A^d peptides could also competitively inhibit the stimulation of antigen specific T cells, thereby implying their affinity/interaction with the intact self I-A molecules.

MATERIALS AND METHODS

Animals: BALB/c (bred at Ellerslie Animal Farm, University of Alberta) and CBA/J mice (Jackson Laboratory, Bar Harbor, Maine) were of 8-12 weeks of age when used.

Antigens and Medium: Antigens used for priming included peptide sequences of I-Aβ^d and I-Aα^d (Table 1) and synthetic peptide antigen EYK(EYA)$_4$ (33, 34). All these peptides were synthesized in this laboratory as described below (33). Purified protein derivative of tuberculin (PPD) (Statens Serum Institute, Copenhagen, Denmark) and Con A (Calbiochem - Behring Corp., LaJolla, CA) were used in cultures as the positive controls in primed

and unprimed T cell responses respectively. RPMI 1640 medium (Gibco) containing 5 x 10^{-5} M 2-mercaptoethanol, 10 mM HEPES, 2mM Glutamine, 5.0 IU/ml Penicillin streptomycin (Gibco) was used with 10% heat inactivated fetal calf serum (BDH).

Synthesis of I-A^d Peptides: Peptides were prepared by the Merrifield solid phase peptide synthesis technique on a Beckman 990C peptide synthesizer as described before (33). All amino acids were protected at the α-amino position with the BOC group and the following side chain blocking groups were used; threonine and serine (benzyl) glutamic acid and aspartic acid (benzyloxy) tryosine (2-bromo benzoxy carbonyl), glutamine and asparagine (4,4-dimethoxybenzhydryl), lysine (2-chlorobenzyloxy carbonyl), histidine and arginine (tosyl) and cysteine (acetamidomethyl). Dicyclohexylcarbodiimide was used as the activating agent and double coupling step of two hours each were performed at each cycle with 2.5 equivalents of BOC amino acids. The BOC groups were removed at each cycle of the synthesis by treatment with 25% TFA in methylene chloride (v/v) for 30 minutes and neutralization was performed by treatment with 5% diisopropylethylamine in methylene chloride (v/v). After completion of the synthesis the Na-BOC group was removed and the peptide resin was further deprotected and cleaved by treatment with HF at 0° for two hours with 10% anisole and 10% DMS (dimethyl sulfide) as scavengers. After removal of the HF under vacuum, the resin was washed with ether and the peptide extracted with TFA. After removal of the TFA

by evaporation the residue was washed with ether, dissolved in water and lyophilized.

The crude peptides were purified by reverse phase high performance liquid chromatography on a semi-preparative synchropak RP-P C18 (250 x 10 mm ID) column, using a linear gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile (1% of second solvent per minute). The composition of peptides was confirmed by amino acid analysis. Peptides were hydrolyzed for 20 hours at 110°C with 6N HCl containing 0.1% phenol in evacuated and sealed tubes. Cystein was converted to cysteic acid before hydrolysis. Analysis was carried out on a Beckman system 6300 amino acid analyzer.

Hybridomas: Poly 18 [Poly EYK(EYA)5] reactive I-A^d restricted T cell hybridoma A.1.1 (35) was generated in this laboratory. Poly 18 reactive T cell hybridoma A.1.1 is readily activated by a synthetic peptide EYK(EYA)4 when presented by fixed APC. Therefore EYK(EYA)4 does not require antigen processing by the APC for the activation of A.1.1 cells (33). A B cell hybridoma TA3 bearing I-$A^{d/k}$/I-$E^{d/k}$ (36) was used as APC in the competitive inhibition experiments. An IL-2 dependent cell line CTL-L (kindly provided by Dr. T. Delovitch, University of Toronto, Canada) was used to assay the IL-2 content.

Monoclonal Antibody: Anti I-A^d monoclonal antibody (MKD6) was used in blocking experiments in ascites form as described before

(34,35). Hybridoma cells producing this antibody were purchased from American Type Culture Collection, Rockville, MD.

Immunization Protocol: For T cell proliferation assay 8-10 week old CBA/J or BALB/c mice were immunized subcutaneously with I-A^d peptides. Mice were injected 25 µg of peptide in 25 µl saline mixed with equal volume of complete Freund's adjuvant (CFA) in each hind footpad. After nine days, mice were killed by $C0_2$ and draining lymph nodes were collected.

Isolation and Culture of Cells: T cells were enriched from primed lymph node cells (LNC) by using nylon wool columns as described earlier (37). Briefly 1 x 10^8 LNC in RPMI + 10% FCS in a 1 ml volume were loaded onto a 10 ml column containing 0.6 g of nylon wool (Terumo, Canada) and incubated for one hour at 37°C in 10% CO_2, after which time the effluent was collected. Cell recovery was about 30%. T cells (5 x 10^5) thus separated were cultured for five days in RPMI medium supplemented with 5 x 10^{-5} M 2-mercaptoethanol, heat inactivated 10% fetal calf serum, glutamine, penicillin streptomycin and hepes buffer in Linbro 96 well flat bottomed plates. Irradiated (4000 rads) spleen cells (10^6 cells/well) from normal syngeneic mice were used as a source of APCs for pulsing with antigen. I-A^d peptides were used at concentrations of 0.125 µM to 125.0 µM in various experiments. At 96 hours 1.0 µCi of ^3H-Thymidine (New England Nuclear) was added per culture for 12-18 hours. Cells were then harvested on a Titretek Harvester Filters.

The filters were dried and counted on a LKB Beta Liquid Scintillation Counter.

Antibody Blocking of T Cell Proliferation Assay: In the blocking studies, a 1/100 final dilution of anti I-A^d monoclonal antibody (MKD6) (as ascites) was added directly to the culture for the entire five day culture period.

Competitive Inhibition Assays:T cell hybridoma A.1.1 (10^5 cells/well) and normal or fixed TA3 (5 x 10^4 cells/well) as APC were cocultured with Poly 18 derived peptide EYK(EYA)4 (40 μg/well) in the presence or absence of I-A^d peptides at varying concentrations (1.25 μM to 375 μM). After 24 hours. supernatants were collected and assayed for IL-2 content in a secondary culture by using IL-2 dependent T cell line CTL-L cells. CTL-L (10^4) cells were cultured for two days with serial dilutions of primary supernatant (starting at 12.5% primary supernatant), and the degree of stimulation was measured by the incorporation of ^{3}H-thymidine (1 μCi/well). Percent Inhibition was calculated using the following formula:

$$\frac{\text{Control CPM (no peptide) - experimental CPM (with peptide)}}{\text{Control CPM (no peptide)}} \times 100$$

For prepulsing experiments TA3 cells were cultured with the antigen Poly 18 derived peptide EYK(EYA)4 (200 μg/ml) for six hours and then washed twice with Hepes buffered balanced salt solutions (HBBSS). 5 x 10^6 cells/ml in HBBSS were treated with a final concentration of 0.05% glutaraldehyde (Fisher Scientific) as

described (38, 39). After half a minute at room temperature, fixation was stopped by addition of an equal volume of 0.2 M lysine (Sigma) in HBBSS. Cells were washed twice with HBBSS and then once with medium before use. Such treated TA3 cells (5 x 10^4) were then cultured with A.1.1 T cell hybridoma (1 x 10^5 cells/well), preincubated with varying concentration of I-A^d peptides (not washed) for 24 hours. Supernatant from these cultures were collected and assayed for IL-2 content as described above. Background cpm of CTL-L cells cultured in medium alone ranged from several hundred to two thousand and in no experiment exceeded this range.

RESULTS

1. I-A^d Peptides Induce in vitro Proliferation in Unprimed Syngeneic BALB/c T Cells.

As an initial approach to examine the tolerant/responsive status of syngeneic mice towards self I-A^d peptides, unprimed BALB/c T cells were separated by nylon wool column from spleens of unimmunized mice and cultured with irradiated syngeneic spleen cells as APCs and various I-A^d peptide fragments as antigens. The result presented in Figure 2.1a shows that among the I-Aβ^d peptides, 1-14 was most stimulatory followed by 84-91, peptides 21-36 and 29-46 were stimulatory to about the same extent but at different concentrations, where as the peptide 62-78 did not stimulate proliferation of T cells. Among the I-Aα^d peptides (Figure 2.1b), 1-17 was most stimulatory, but the proliferative response towards this

peptide was far less than towards I-Aβ^d peptides. Other I-Aα^d peptides eg. 26-36, 51-69 and 71-84 were less stimulatory than 1-17 but still these peptides stimulated T cell proliferation above background level. Inspired by the immunological phenomenon of alloreactivity we examined the response of unimmunized BALB/c T cells towards I-Aβ^k peptide 1-14. To our surprise there was almost no stimulation by I-Aβ^k 1-14 peptide (Figure 2.1a). We also examined the response of unprimed (naive) BALB/c T cells towards unrelated I-A^d restricted antigens, beef insulin (34) and EYK(EYA)$_4$ (33) and they were found to be nonstimulatory (Figure 2.1b).

2. I-A^d Peptides Induce in vitro Proliferation in Peptide Primed BALB/c T Cells.

To further establish that I-A^d peptide fragments were immunogenic in syngeneic mice, BALB/c mice were footpad primed with various I-A^d peptides in CFA. After nine days T cells from popliteal lymph nodes of these animals were nylon wool separated and cultured with various peptides and irradiated syngeneic spleen cells as APCs. I-Aβ^d 29-46 peptide gave the highest response as compared to other peptides tested (Table 2.2a, 2.2b). Response to peptides I-Aβ^d 9-20 and 21-36 was higher with the primed T cells as compared to the response of unprimed T cells, whereas response to peptides I-Aβ^d 1-14 and 84-91 was similar to that of unprimed T cells (Table 2.2a). Interestingly, very little stimulation of T cell proliferation was observed by the peptide I-Aβ^d 62-78. Among the I-Aα^d peptides (Table 2.2b), peptides 1-17 and 71-84 were immunogenic while peptide 26-36 did not stimulate the T cell

peptide was far less than towards I-Aβ^d peptides. Other I-Aα^d peptides *i.e.* 26-36, 51-69 and 71-84 were less stimulatory than 1-17 but still these peptides stimulated T cell proliferation above background level. Inspired by the immunological phenomenon of alloreactivity we examined the response of unimmunized BALB/c T cells towards I-Aβ^k peptide 1-14. To our surprise there was almost no stimulation by I-Aβ^k 1-14 peptide (Figure 2.1a). We also examined the response of unprimed (naive) BALB/c T cells towards unrelated I-A^d restricted antigens, beef insulin (34) and EYK(EYA)4 (33) and they were found to be nonstimulatory (Figure 2.1b).

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proliferation at any of the concentrations tested. Peptide 51-69 stimulated the T cell proliferation only at high concentration. As expected a considerable response towards the allo MHC II peptide I-Aβ^k 1-14 was observed in primed T cells (Table 2.2b) as compared to the unprimed T cells (Figure 2.1a).

3. T Cells from I-A^d Peptides Primed BALB/c Mice do not Proliferate in Response to Unrelated Antigens.

T cells from mice individually primed with peptides I-Aβ^d 1-14, 9-20, 21-36, 29-46 and I-Aα^d 71-84 in CFA were cultured with unrelated but I-A^d restricted antigens EYK(EYA)$_4$ (33, 39) and beef insulin (34) in the presence of irradiated syngeneic spleen cells as APCs. Both of the unrelated antigens failed to induce T cell proliferation (Table 2.3) at all the concentrations tested. Therefore the stimulation of T cells by I-A peptides was specific and not due to a generalized polyclonal stimulation.

4. Monoclonal Antibody to I-A^d (MKD6) Blocks the in vitro Proliferation of Peptide Primed BALB/c T Cells in Response to I-A^d Peptides.

In order to confirm that BALB/c T cell responses to peptide fragments of syngeneic I-A molecules require involvement of Ia molecules on the APCs for presentation, monoclonal anti-I-A^d antibodies (MKD6) were used. T cells were obtained from BALB/c mice primed with I-A^d peptides. These peptide primed T cells were cultured with the priming peptide and irradiated syngeneic spleen cells in the presence or absence of MAb to I-A^d (MKD6). Response of

T cells to almost all of the I-Aα^d and I-Aβ^d peptides except I-Aβ^d 21-36 peptide was completely abrogated by the addition of anti-I-A^d monoclonal antibody MKD6 (Table 2.4). The concentration of MKD6 MAb used in this experiment was not toxic for the T cells as their viability remained the same in the presence or absence of MKD6 Ab. The viability was determined by trypan blue exclusion test.

5. I-A^d Peptides Induce in vitro T Cell Proliferation in Peptide Primed Allogeneic CBA/J T Cells.

To determine if the peptide fragments of I-A^d were immunogenic in allogeneic strain, CBA/J (H-2^k) mice were footpad primed with both I-Aβ^d and I-Aα^d peptides in CFA (32). After nine days, T cells from the popliteal lymph nodes of these animals were separated by nylon wool column and cultured with irradiated CBA/J spleen cells as APCs and various I-A^d peptide fragments. Among I-Aα^d peptides, 1-14 was most stimulatory. In contrast to the response in the syngeneic BALB/c mice (Table 2.2a), peptide I-Aβ^d 62-78 was stimulatory where as peptide 9-20 was not. Peptides I-Aβ^d 21-36, 28-[illegible]4-91 also stimulated the *in vitro* proliferation of primed CBA/J T cells (Table 2.5a). Among the I-Aα^d peptides, 1-17 was most stimulatory where as 51-69 being the least (Table 2.5b). Peptide 26-36 also showed considerable stimulation as compared to the syngeneic BALB/c mice (Table 2.5b compared to Table 2.2b). Peptide I-Aβ^k 1-14 also stimulated the *in vitro* proliferation of syngeneic CBA/J T cells (Table 2.5b).

6. I-A^d Peptides Inhibit the Presentation of Antigens to T Cell Hybridoma by Binding to Syngeneic APC.

To determine the affinity of I-A^d peptides towards intact self I-A molecule, competitive inhibition experiments were performed. The rationale was that if the self I-A peptides have an affinity for self I-A, they will compete with antigens in the activation of T cell hybridoma when added to the culture. I-A^d restricted Poly 18 specific T cell hybridoma A.1.1 were cultured with B cell hybridoma cells (TA3) as APC and the respective antigen in the presence or absence of I-A^d peptides (Figure 2.2a, 2.2b). Supernatant from these cultures were tested for IL-2 activity in a secondary culture using CTL-L cells proliferation as an indicator. Various I-Aβ^d peptides inhibited the stimulation of A.1.1 T cells by up to 90% (Figure 2.2a) and I-Aα^d peptides inhibited the response up to 50% (Figure 2.2b). As described elsewhere EYK(EYA)$_4$ peptide is efficiently presented by fixed APC (33) and similar inhibition was observed when fixed APC were used in these experiments. The I-A^d peptides did not affect the viability of T cells in culture as judged by trypan blue exclusion test at any concentrations used in inhibition experiments. Therefore, the inhibition of activation of T cells by these peptides is not due to nonspecific toxicity. As a control we examined the effect of hen egg lysozyme, an IA^k binding antigen, on the activation of T cells and found almost no (<10%) inhibition (Figure 2.2a).

To investigate whether the inhibition of activation of A.1.1 T cells by I-A^d peptides was due to competition between the antigen and the inhibitor peptides for the antigen binding site on

intact MHC or due to the competition for recognition by T cell receptor, a prepulsing experiment was performed. Antigen prepulsed and glutaraldehyde fixed TA3 cells were cultured with T cells prepulsed with I-A^d peptides, I-A^d peptides were present all through the primary culture. Activation of T cells was determined by IL-2 secretion. In this system, almost no inhibition in T cell activation was observed even in the presence of optimum inhibitory concentration of I-A^d peptides (Table 2.6). This suggests that the inhibition of T cell activation by I-A^d peptides is because of the competition at the antigen binding site on the intact MHC molecule and not due to the competition for recognition by the T cell receptor.

DISCUSSION

The results presented in this paper demonstrate that strong T cell proliferative responses can be generated against peptide fragments of I-A molecules in both syngeneic and allogeneic mice (32). Our results viz. the stimulation of unimmunized syngeneic T cells by I-A peptides are unexpected and suggest the endogenous priming of these T cells by self I-A peptides *in vivo*. Further evidence for this comes from the fact that T cells from the unprimed allo mice failed to be stimulated. Direct evidence for functional self protein/Ia molecule complexes *in vivo* has recently been found (14-16, 40) which may support our hypothesis of *in vivo* priming by self I-A. In Class I MHC systems, peptides derived from the MHC have been found to functionally bind MHC molecules (40-43). In fact we showed some time ago that Class I H-2K^b peptides are presented by

allo Class II MHC molecules (43). Our data on the stimulation of unprimed T cells by self MHC Class II peptides suggests that MHC peptides are presented by the self MHC. This is analogous to the presentation of other self antigens by the MHC antigens (14-16).

A comparison of the titration of I-A^d peptide antigens in our experiments against a fixed number of T cells gave a tri phasic dose response curve. In the first phase lower proliferative response was observed corresponding to the suboptimal dose of antigen in the culture. Maximum response was found in the second phase corresponding to the optimal concentration of antigen for the fixed number of T cells used in the experiment. Further increase in the dose of antigen towards a supraoptimal concentration led to a decrease in the proliferative response in the third phase. Similar kind of observation has been made in studies with other antigen systems (44, 45). The reason for lower response at higher antigen dose could be either the induction of unresponsive state of T cells by some intercellular regulatory signals or a competition between I-A^d peptides and MHC molecules for recognition by the TCR. Since I-$A\beta^d$ 1-14 peptides were most immunogenic we used the corresponding allo I-$A\beta^k$ 1-14 peptide as a control in these studies. In addition various I-A^d peptides served as internal controls. All I-A peptides used here were non-toxic and non-mitogenic to the T cells at all the concentrations used in the proliferation assay. These self I-A peptide reactive T cells do not lead to an autoimmune disease state in the normal mice, which can be explained on the basis of either the presence of insufficient density of self I-A peptides (30, 31),

difference in the conformation of these peptides when bound to MHC or the presence of regulatory cells (46) *in vivo* .

The proliferative response of primed syngeneic T cells towards self I-A peptides was expected to be increased as compared to the unprimed T cells because of clonal expansion of reactive T cells upon immunization (32). This was actually found with the I-Aβ^d peptides 9-20, 21-36, 29-46 and I-Aα^d peptides 71-84. The response to the peptides I-Aβ^d 1-14, 84-91 and I-Aα^d 1-17, 51-69 was similar in both unprimed and primed T cells, which might suggest that either these peptides did not selectively increase the ratio of reactive T cells upon immunization or the T cells reactive to these peptides were already primed due to endogenous presentation of these peptides. Results with the peptide I-Aα^d 26-36 were curious, where unprimed T cells were stimulated but primed T cells were not. One of the possibilities for this kind of behaviour is the induction of regulatory/inhibitory cells in syngeneic mice, however, we have no evidence for it at the present time. This peptide induced strong proliferative responses in primed allo T cells.

The proliferative response of T cells towards self I-A^d peptides seems to be due to the presentation of these peptides by the intact I-A molecule, as is evident by the anti I-A^d MAb blocking experiment. Only in one case, e.g. with I-Aβ^d 21-36 peptide, the response was not blocked by anti I-A^d antibody. One possibility seems to be the presentation of this peptide by I-E^d molecule. In line with the MAb blocking experiment which suggested the presentation of I-A^d peptides by self intact I-A^d molecule, the affinity/interaction of I-A^d peptides with self intact I-

A^d molecule was further confirmed by competitive inhibition experiment. The inhibition of stimulation of antigen specific T cells by class II MHC peptides has been used to demonstrate the binding and interaction of these peptides with intact self MHC II molecules (47). In contrast to the report by Rosloneic *et al.* (47) for I-A^k, I-$A\beta^d$ peptides were found to be more inhibitory than I-$A\alpha^d$ peptides in our experiments. This difference could be reasoned on the basis of the selection of peptide sequences and the haplotype difference. In our case, however, the high immunogenicity of I-$A\beta^d$ peptides is clearly reflected by the high affinity of these peptides for intact self MHC molecule. The only crucial and contradictory peptide seemed to be the I-$A\beta^d$ 62-78 peptide. The maximum inhibition of the stimulation of antigen specific T cells by this peptide was at lowest concentration used (eg. 1.25 μM), suggesting its high affinity for intact MHC II molecule used and yet it did not stimulate the proliferation of syngeneic T cells. The reason for this nonresponsiveness could be the self tolerance against the peptide I-$A\beta^d$ 62-78 or the induction of inhibitory/regulatory cells. Alternatively, it may be the only epitope which gets processed and presented to T cells during thymic or peripheral tolerance induction phase while other epitopes on I-A are not readily available. In addition the possibility remains that thymic dendritic cells process antigen differently then thymic epithelial cells. If this applies to self MHC antigens then it will shape the peripheral T cell repertoire to recognize some self epitopes and delete others.

Further evidence for the competitive inhibition by I-A^d peptides being at the antigen binding site on MHC molecule and not

at recognition by TCR came from the experiment using antigen prepulsed and glutaraldehyde fixed APCs. Glutaraldehyde inhibits the cell's metabolic processes, presumably by cross linking surface molecules and thereby preventing cellular exchange with the environment. Our hypothesis about the inhibition of stimulation of antigen specific T cells by I-A^d peptides was based on a competition or peptide exchange mechanism for the relevant antigen binding site on MHC molecule. By glutaraldehyde fixation of the antigen prepulsed APCs, the antigenic peptides bound to intact MHC molecule would not be replaced by the competitor peptide and hence the competition could take place only at the level of recognition by T cell receptor. The competition by I-A^d peptides for recognition by TCR was further maximized by prepulsing the T cells with I-A^d peptides. The results, however, showed no inhibition in such an experiment by any of the I-Aβ^d and I-Aα^d peptides, further suggesting the competitive inhibition by I-A^d peptides to occur at the antigen binding site on MHC molecule. Although in our experiments most of the α and β peptides seemed to inhibit the stimulation of antigen specific T cells by binding to intact MHC molecule, this observation should not be taken to imply that the peptide sequences from entire region of I-A molecule would have affinity for intact MHC molecule. Rather, the random selection of peptide sequences from polymorphic areas of α and β chains of I-A^d molecule may have coincidentally selected the peptide sequences with high affinity for intact self I-A molecule. Experiments to test this hypothesis require synthesis of peptides related to the non polymorphic region. These experiments are currently underway.

Despite the high affinity of these I-A^d peptides for self I-A^d molecule, generation of strong T cell proliferative response against them (except peptide I-$A\beta^d$ 62-78) was surprising and raised questions about the phenomenon of self tolerance. Various possibilities can be considered to address this observation. During thymic differentiation the self MHC peptides are not presented on the thymic APCs in this form and hence thymocytes reacting with self I-A peptides in context of self MHC molecule are not deleted. The region corresponding to the peptide I-$A\beta^d$ 62-78 may have been exposed to the developing thymocytes and hence T cells reactive to this region of I-A molecule are deleted. The residue 65-67 on the I-Aβ chain have been implicated in contacting the T cell receptor in the recognition of pre-formed MHC-peptide complexes (48). The fact that several potentially powerful immunodominant determinants do not make an impact on the diversity of the T cell repertoire suggests that mechanisms have evolved to allow many self reactive T cells, directed against subdominant or cryptic determinants to escape elimination through clonal deletion. Partial tolerance to self MHC Class II antigens may positively influence the T cell repertoire, and tolerance to all self epitopes may not be necessary for the prevention of autoimmune disease (49). In conclusion, our results suggest that certain MHC class II derived peptides with high affinity for self MHC can elicit potent T cell responses. On the other hand T cells do undergo negative selection or elimination process in order to establish self tolerance to some epitopes on class II MHC molecules.

Table 2.1

Various synthetic peptides used in these studies

Synthetic Peptide	Region on intact I-A molecule	Sequence of Peptide
I-Aβ^d	β_1 domain	
1-14	amino terminal HV1	G N S E R H F V V Q F K G E
9-20	HV1	V Q F K G E C Y Y T N G
21-36	-	T Q R I R L V T R Y I Y N R E E
29-46	-	R Y I Y N R E E Y V R Y D S D V G E
62-78	HV2	N S Q P E I L E R T R A E V D T A
84-91	carboxy terminal HV3	E G P E T S T S
I-Aα^d	α_1 domain	
1-17	amino terminal HV1	E D D I E A D H V G F Y G T T V Y
26-36	-	Y T H E F D G D E L F
51-69	HV3	E F G Q L I L F E P Q G G L Q N I A A
71-84	carboxy terminal HV	K H N L G I L T K R S N F T
I-Aβ^k	β_1 domain	
1-14	amino terminal HV1	G N S E R H F V H Q F Q P F

Table 2.2a

In vitro T cell proliferative response of I-Aβ^d peptides primed syngeneic BALB/c mice

Priming Antigen[b] I-A Peptides	Antigens in Culture	Concentration of Ag. in Culture (μM)	^{3}H thymidine incorporation CPM ± S.D.
I-Aβ^d 1-14	-	-	4,174 ± 1,992
	PPD	a	34,427 ± 298
	I-Aβ^d 1-14	1.25	57,819 ± 2,046
		12.5	53,909 ± 8,249
I-Aβ^d 9-20	-	-	1,696 ± 371
	PPD	a	10,438 ± 76
	I-Aβ^d 9-20	1.25	16,178 ± 829
		12.5	22,961 ± 5,341
I-Aβ^d 21-36	-	-	3,496 ± 581
	PPD	a	29,496 ± 6,650
	I-Aβ^d 21-36	1.25	39,533 ± 6,024
		12.5	64,324 ± 8,174
I-Aβ^d 29-46	-	-	3,269 ± 146
	PPD	a	71,634 ± 5,176
	I-Aβ^d 29-46	1.25	45,316 ± 5,367
		12.5	86,186 ± 4,457
I-Aβ^d 62-78	-	-	5,149 ± 690
	PPD	a	58,188 ± 4,822
	I-Aβ^d 62-78	1.25	6,620 ± 479
		12.5	4,979 ± 465
		125.0	6,179 ± 1,642
I-Aβ^d 84-91	-	-	3,750 ± 1,615
	PPD	a	98,978 ± 14,380
	I-Aβ^d 84-91	1.25	29,400 ± 5,265
		12.5	23,801 ± 5,370

a. PPD was used at a concentration of 100 μg/ml.

b. Mice were immunized with antigens (25 μg) in CFA in both hind footpads.

Table 2.2b

In vitro T cell proliferative response of I-A_α^d peptides primed BALB/c mice

Priming Antigen[b] I-A Peptides	Antigens in Culture	Concentration of Ag. in Culture (μM)	^{3}H thymidine incorporation CPM ± S.D.
I-A_α^d 1-17	-	-	5,550 ± 187
	PPD	a	129,242 ± 13,155
	I-A_α^d 1-17	1.25	36,778 ± 4,465
		12.5	44,475 ± 4,153
I-A_α^d 26-36	-	-	2,906 ± 479
	PPD	a	43,063 ± 7,279
	I-A_α^d 26-36	1.25	2,833 ± 749
		12.5	3,235 ± 396
I-A_α^d 51-69	-	-	4,432 ± 300
	PPD	a	530 ± 11,963
	I-A_α^d 51-69	1.25	[illegible] ± 751
		12.5	[illegible] ± 1770
		125.0	[illegible] ± 6,012
I-A_α^d 71-84	-	-	505 ± 192
	PPD	a	41,658 ± 4,012
	I-A_α^d 71-84	1.25	14,151 ± 2,709
		12.5	6,617 ± 793
I-A_β^k 1-14 [c]	-	-	3,392 ± 1,150
	PPD	a	38,650 ± 2,593
	I-A_β^k 1-14	1.25	28,903 ± 2,963
		12.5	16,665 ± 479

a. PPD was used at a concentration of 100 μg/ml.
b. Mice were immunized with antigens (25 μg) in CFA in both hind footpads.
c. Allogeneic peptide.

Table 2.3

Proliferative Response of I-A peptides primed BALB/c T cells to other antigens presented by I-A^d bearing APC's

Priming Antigen[a]	Beef Insulin[b]		EYK(EYA)$_4$[b]	
		^{3}H-Tdr Incorporation		^{3}H-Tdr Incorporation
I-A peptides	Conc.(μM)	CPM(x10^{-3})	Conc.(μM)	CPM(x10^{-3})
Unprimed Control		0.0	2.2	0.0 2.2
I-Aβ^d (1-14)	62.5	3.0	125.0	2.4
	6.25	2.3	12.5	1.8
	0.625	2.1	1.25	1.9
I-Aβ^d (9-20)	62.5	2.2	125.0	0.7
	6.25	3.4	12.5	2.1
	0.625	3.4	1.25	1.9
I-Aβ^d (21-36)	62.5	2.1	125.0	2.2
	6.25	2.2	12.5	2.2
	0.625	2.1	1.25	2.2
I-Aβ^d (29-46)	62.5	3.0	125.0	3.0
	6.25	3.2	12.5	3.2
	0.625	1.9	1.25	2.1
I-A$_\alpha^d$ (71-84)	62.5	2.0	ND	ND
	6.25	1.8	ND	ND
	0.625	1.5	ND	ND

a. Mice were primed with the I-A peptides (25 μg) in CFA in the hind footpads.
b. I-A peptide primed T cells were cultured with these antigens as in Table 2.2.

Table 2.4

Inhibition of proliferation of I-A peptides primed BALB/c T cells with monoclonal antibody to I-A^d

Priming Antigen I-A peptides [a]	Antigens in culture Conc. (μM)	MAb MKD6[b]	Incorporation of ^{3}H-Tdr CPM ± S.D. ($x10^{-3}$)
I-$A\beta^d$	12.5	-	33.2 ± 4.5
(1-14)	12.5	+	1.1 ± 0.8
I-$A\beta^d$	12.5	-	42.1 ± 6.9
(9-20)	12.5	+	4.4 ± 0.2
I-$A\beta^d$	12.5	-	56.8 ± 4.8
(21-36)	12.5	+	47.6 ± 9.0
I-$A\beta^d$	12.5	-	62.0 ± 3.1
(29-46)	12.5	+	4.4 ± 4.1
I-$A\beta^d$	12.5	-	8.8 ± 1.2
(62-78)	12.5	+	2.2 ± 0.2
I-$A\beta^d$	12.5	-	15.5 ± 3.2
(84-91)	12.5	+	2.2 ± 1.1
I-$A\alpha^d$	12.5	-	24.3 ± 1.6
(1-17)	12.5	+	2.2 ± 0.4
I-$A\alpha^d$	12.5	-	2.2 ± 0.3
(26-36)	12.5	+	0.4 ± 0.0
I-$A\alpha^d$	12.5	-	2.2 ± 0.2
(51-69)	12.5	+	0.2 ± 0.0
I-$A\alpha^d$	12.5	-	22.2 ± 3.4
(71-84)	12.5	+	2.2 ± 0.2

a. Mice were immunized with I-A peptides (25 μg) in CFA in both hind

b. MAb (MKD6) was used at a final concentration of 1/100 (in ascites

Table 2.5a

In vitro proliferation of T cells from allogeneic CBA/J (H-2 ^{k}) mice primed with I-Aβ^{d} peptides

Priming Antigen[b] I-A Peptides	Antigens in Culture	Concentration of Antigen in Culture (μM)	^{3}H thymidine incorporation CPM ± S.D.
I-Aβ^{d} 1-14	-	-	2,205 ± 549
	PPD	a	67,206 ± 19,629
	I-Aβ^{d} 1-14	0.625	15,174 ± 3,518
		1.25	22,179 ± 2,766
		12.5	51,899 ± 6,508
I-Aβ^{d} 9-20	-	-	4,082 ± 76
	PPD	a	75,983 ± 5,293
	I-Aβ^{d} 9-20	1.25	4,802 ± 415
		12.5	11,897 ± 595
		125.0	9,815 ± 1,143
I-Aβ^{d} 21-36	-	-	3,235 ± 738
	PPD	a	38,597 ± 2,614
	I-Aβ^{d} 21-36	0.625	6,377 ± 765
		1.25	12,548 ± 602
		12.5	29,551 ± 7,192
		125.0	16,639 ± 1,481
I-Aβ^{d} 29-46	-	-	1,439 ± 359
	PPD	a	35,899 ± 5,977
	I-Aβ^{d} 29-46	0.625	10,799 ± 593
		1.25	38,253 ± 2,229
		12.5	22,808 ± 3,946
		125.0	17,114 ± 2,453
I-Aβ^{d} 62-78	-	-	955 ± 190
	PPD	a	28,841 ± 7,721
	I-Aβ^{d} 62-78	0.625	8,724 ± 682
		1.25	5,844 ± 613
		12.5	14,905 ± 3,617
		125.0	4,981 ± 935
I-Aβ^{d} 84-91	-	-	2,970 ± 472
	PPD	a	34,664 ± 3,784
	I-Aβ^{d} 84-91	0.625	2,779 ± 745
		1.25	2,699 ± 362
		12.5	6,311 ± 957

a. PPD was used at a concentration of 100 μg/ml.

b. Peptide antigen (25 μg) in CFA was injected in both hind footpads of mice.

Table 2.5b

In vitro proliferation of T cells
from allogeneic CBA/J (H-2^k) mice primed with IA$_\alpha^d$ peptides

Priming Antigen[b] I-A Peptides	Antigens in Culture	Concentration Antigen in Culture (μM)	^{3}H thymidine incorporation CPM ± S.D.
I-A$_\alpha^d$ 1-17	-	-	1,549 ± 385
	PPD	a	38,198 ± 1,276
	I-A$_\alpha^d$ 1-17	0.625	26,625 ± 1,166
		1.25	52,543 ± 6,326
		12.5	4,886 ± 1,254
		125.0	25,023 ± 3,784
I-A$_\alpha^d$ 26-36	-	-	2,631 ± 192
	PPD	a	36,905 ± 7,126
	I-A$_\alpha^d$ 26-36	0.625	25,814 ± 4,618
		1.25	44,527 ± 4,983
		12.5	19,107 ± 1,408
		125.0	7,153 ± 1,231
I-A$_\alpha^d$ 51-69	-	-	1,561 ± 275
	PPD	a	61,553 ± 9,110
	I-A$_\alpha^d$ 51-69	0.625	4,592 ± 1,370
		1.25	13,139 ± 2,422
		12.5	9,372 ± 1,384
		125.0	3,237 ± 226
I-A$_\alpha^d$ 71-84	-	-	1,752 ± 226
	PPD	a	36,199 ± 1,993
	I-A$_\alpha^d$ 71-84	0.625	25,888 ± 2,102
		1.25	5,175 ± 2,358
		12.5	19,874 ± 2,278
		125.0	11,259 ± 1,978
I-A$_\beta^k$ 1-14 [c]	-	-	1,963 ± 715
	PPD	a	54,639 ± 6,310
	I-A$_\beta^k$ 1-14	0.625	35,636 ± 2,469
		1.25	9,806 ± 1,346
		12.5	3,394 ± 1,016

a. PPD was used at a concentration of 100 μg/ml.
b. Peptide (25 μg) in CFA was injected in both hind footpads of mice.
c. Syngeneic peptide.

Table 2.6a

I-A^d peptides do not inhibit the activation of A.1.1. T cell hybridoma by antigen prepulsed and fixed APC

T cell Hybridoma[a]	APC[b]	Inhibitory I-A Peptides (125 μm)[c]	^{3}H-Tdr Incorporation by CTL-L Cells (CPM ± S.D.)[d]
A.1.1.	TA3 alone	-	1,090 ± 345
A.1.1.	Antigen Prepulsed TA3	-	132,015 ± 1,577
A.1.1.	Fixed TA3 + Antigen 40 μg	-	100,526 ± 2,204
A.1.1.	Antigen Prepulsed and Fixed TA3	-	122,657 ± 14,308
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 1-14	120,320 ± 1,898
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 9-20	127,504 ± 13,650
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 21-36	118,828 ± 1,205
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 29-46	119,563 ± 12,169
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 62-78	125,547 ± 1,099
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aβ^d 84-91	120,658 ± 5,478

A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aα^d 1-17	109,635 ± 5,385
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aα^d 26-36	120,369 ± 5,368
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aα^d 51-69	115,728 ± 7,361
A.1.1.	Antigen Prepulsed and Fixed TA3	I-Aα^d 71-84	121,315 ± 1,997

a. Poiy 18. Poly EYK(EYA)$_5$ specific T cell hybridoma A.1.1. was used at 1 x 10^5 cells/well. These cells respond to EYK(EYA)$_4$ peptide and no further processing of this peptide is required for stimulation (39).

b. TA3 B cell hybridoma cells were used as APC at 5 x 10^4 cells/well. TA3 cells were prepulsed with EYK(EYA)$_4$ antigen at 200 µg/ml for 2 hours, washed and fixed with glutaraldehyde as discussed in Material and Methods.

c. Various I-Aα^d and I-Aβ^d peptides at 125 µm concentration were added to A.1.1. T cell hybridoma 24 hours before the addition of TA3 cells.

d. IL-2 generated by A.1.1. cells were measured by the ^{3}H-Tdr incorporation by CTL-L cells following incubation with supernatant from the A.1.1. cells as described in Materials and Methods

Table 2.6b

Activation of A.1.1. T cell hybridoma is inhibited by fixed APCs prepulsed with I-A^d peptides

T cell Hybridoma[a]	Fixed APCs prepulsed with I-A peptides[b]	Antigen in culture[c]	^{3}H-Tdr incorporation by CTL-L cells (CPM ± S.D.)[d]
A.1.1.	Fixed APC Only	No Antigen	233 ± 90
A.1.1.	Fixed APC Only	EYK(EYA)$_4$	66,967 ±1,918
A.1.1.	I-Aβ^d 1-14	EYK(EYA)$_4$	15,165 ±1,933
A.1.1.	I-Aβ^d 9-20	EYK(EYA)$_4$	23,971 ±4,417
A.1.1.	I-Aβ^d 62-78	EYK(EYA)$_4$	15,064 ±1,445
A.1.1.	I-Aα^d 1-17	EYK(EYA)$_4$	26,408 ±2,413
A.1.1.	I-Aα^d 71-84	EYK(EYA)$_4$	23,644 ±2,288

a Same as in Table 2.6a.

b TA3 B cell hybridoma cells were used as APCs at 5 x 10^4 cells/well. TA3 cells were fixed with glutaraldehyde as described in Materials and Methods and prepulsed with I-A^d peptides (125 µM) or medium alone for 4 hours before addition of antigen and T cell hybridoma cells.

c EYK(EYA)$_4$ peptide was used as antigen at a concentration of 50 µg/ml.

d Same as in Table 2.6a.

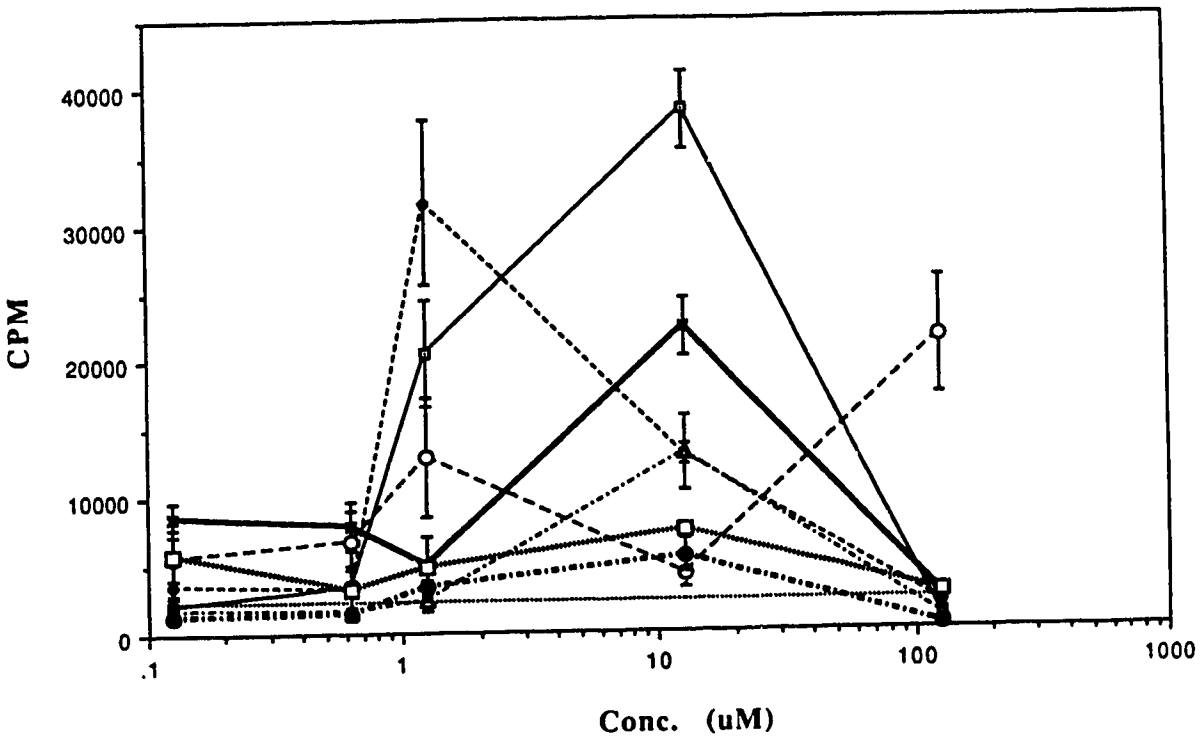

Figure 2.1a ***Proliferation of unprimed BALB/c T cells by I-Aβ^d peptides.***

T cells were obtained from spleens of unprimed BALB/c mice and cultured with syngeneic APCs plus antigens as described in Materials and Methods. (—▫—) I-Aβ^d 1-14, (--△---) I-Aβ^d 9-20, (—×—) I-Aβ^d 21-36, (--o---) I-Aβ^d 29-46, (-·-·●·-·-), I-Aβ^d 62-78, (---◇---) I-Aβ^d 84-91, (·····□·····) I-Aβ^k 1-14 at various concentrations and (·····) control (no antigen).

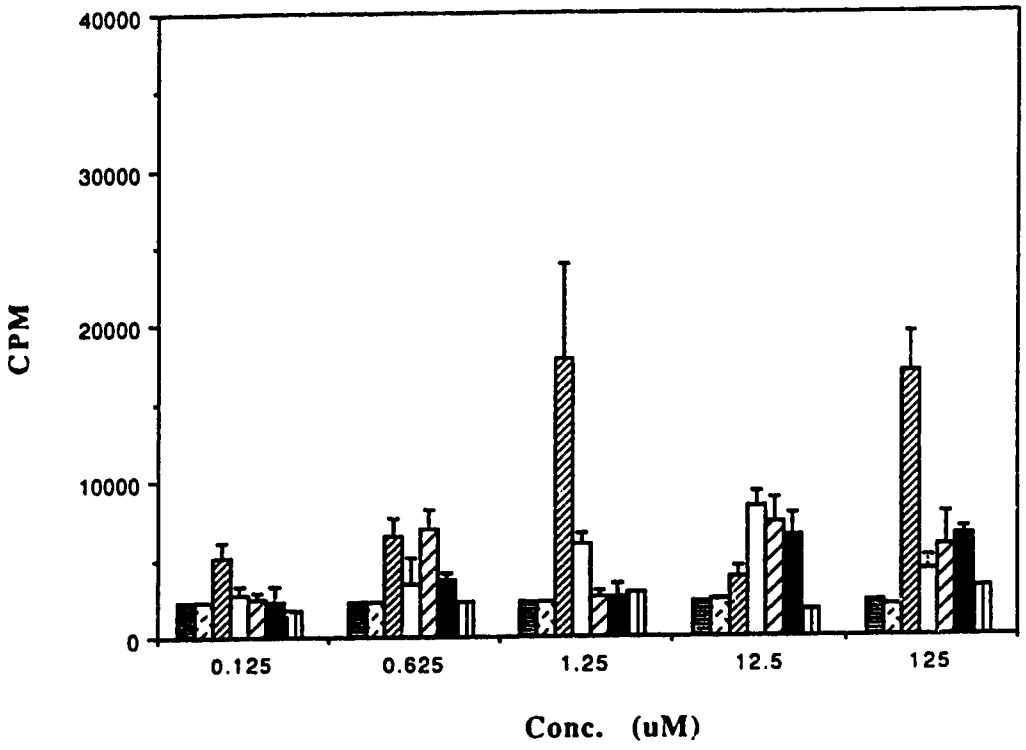

Figure 2.1b ***Proliferation of unprimed BALB/c T cells by I-Aα^d peptides.***

See Figure 2.1a legend for details. (▨) I-Aα^d 1-17, (❑) I-Aα^d 26-36, (▨)I-Aα^d 51-69, (■) I-Aα^d 71-84 and (▤) no antigen (control), (▨) beef insulin (control), (▥) EYK(EYA)$_4$ (control), were used at various concentrations.

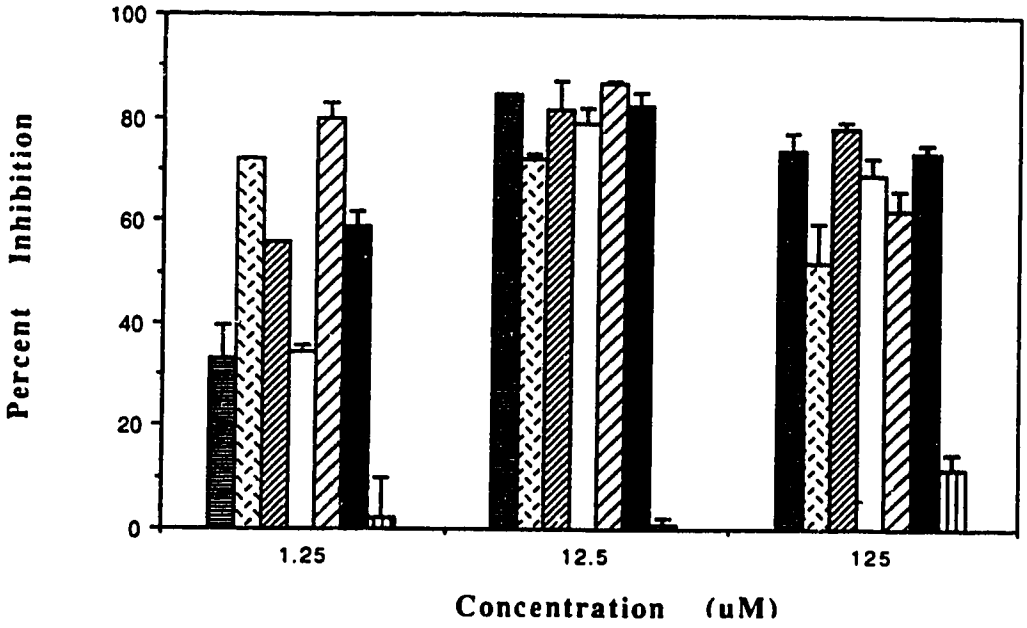

Figure 2.2a ***Inhibition of stimulation of EYK(EYA)4 reactive T cell hybridoma, A.1.1 by I-Aβ^d peptides***

(▤) I-Aβ^d 1-14, (▨) I-Aβ^d 9-20, (▨) I-Aβ^d 21-36, (❑) I-Aβ^d 29-46, (▨) I-Aβ^d 62-78, (■) I-Aβ^d 84-91 and (▥) control I-A^k restricted antigen hen egg lysozyme (HEL) were used at various concentrations. The magnitude of the response of T cells as measured by ^{3}H-Tdr incorporation by CTLL and described in Materials and Methods was 2,008 ± 657 and 150,342 ± 2,651 CPMs in the absence and the presence of antigen respectively.

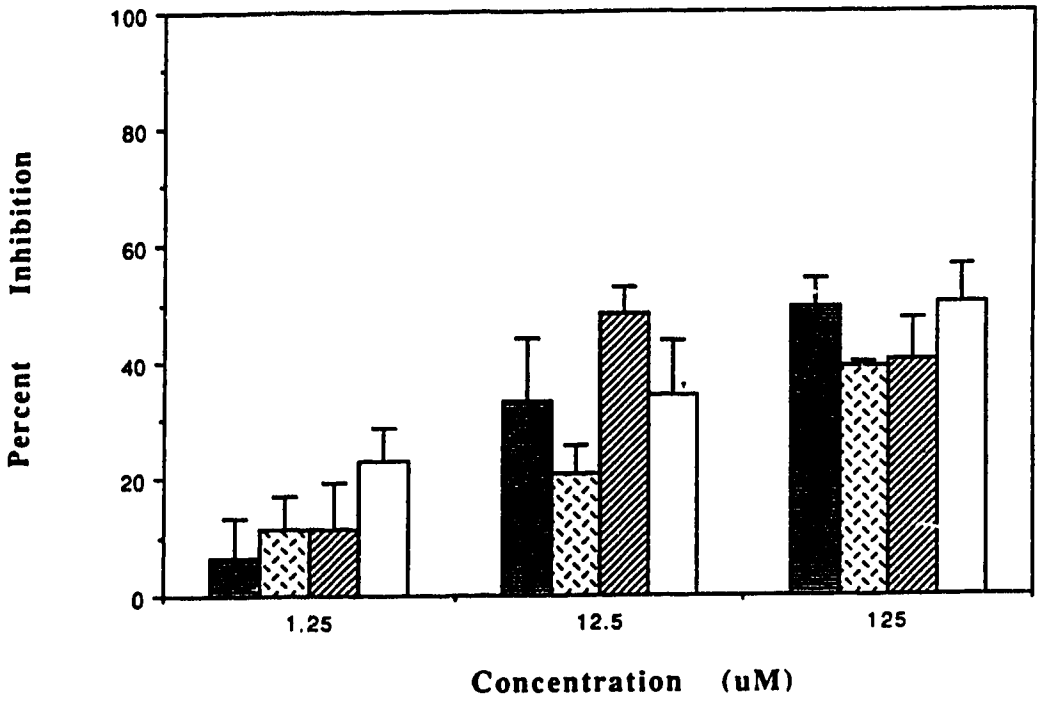

Figure 2.2b ***Inhibition of stimulation of EYK(EYA)$_4$ reactive T cell hybridoma, A.1.1 by I-Aα^d peptides.***
(▤) I-Aα^d 1-17, (▦) I-Aα^d 26-36, (▨) I-Aα^d 51-69 and (❑) I-Aα^d 71-84 peptides were used at various concentrations. The magnitude of uninhibited response was the same as in Figure 2.2a.

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CHAPTER III

LOSS OF TOLERANCE TO AN IMMUNODOMINANT REGION OF SELF MHC CLASS II MOLECULE WITH AGE

INTRODUCTION

Self tolerance in the immune system is essential for the self/nonself discrimination and also for the maintenance of integrity of self. The general mechanisms which have been proposed for achieving self tolerance are clonal deletion (1-3) or clonal anergy (4-8) of self reactive T cell clones. These mechanisms may operate at various stages of T cell development. The self antigens involved in tolerance induction are combinations of self peptides/MHC molecules which requires the processing and presentation of self proteins in context of self MHC molecules. Direct evidence for the existence of self peptides/self MHC molecule complexes comes from functional studies (9-12) as well as from studies where naturally processed peptides were acid eluted from affinity purified class II molecules (13). An indirect evidence for processing and presentation of self MHC class II molecules *in vivo* has come from our previous studies (14), where unprimed naive T cells from BALB/c mice seemed to be primed to self I-A^d peptides. Although self tolerance is necessary to

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prevent autoimmunity, overwhelming T cell depletion has to be avoided in order to provide functionally diverse T cell repertoire. MHC class II molecules are critical self molecules playing central role in the induction and regulation of an immune response (15). They are also present in the thymus at the time of negative and positive selection required for the acquisition of T cell repertoire during development.

Using a number of peptides from the α helical and β pleated region of the proposed antigen binding groove of the MHC class II (I-A) molecules (ie. polymorphic amino terminal domain of α and β chains), we (14) and others (16) have examined the T cell responses to self I-A molecule. We recently reported that a number of self I-A peptides bind to self MHC class II molecules and stimulate proliferation of syngeneic T cells. However, one of the non stimulatory peptides I-Aβ^d 62-78, bound to I-A^d with high affinity but failed to stimulate a T cell response presumably because of self tolerance as it was immunogenic in allogeneic H-2^k (CBA/j) mice. The peptide representing the region I-Aβ 62-78 has also been implicated in contacting the TCR in the recognition of MHC-peptide complexes (17). The hypothetical model of ag binding site of MHC class II molecules (18) suggests the region I-Aβ 62-78 to be on the α helix forming the wall of ag binding groove and be exposed to TCR. It can be speculated that the region of I-A corresponding to I-Aβ^d 62-78 may have been exposed to developing thymocytes and T cells reactive to this region are deleted.

In the present study we have examined the development and progression of T cell reactivity and tolerance to self I-A peptides

in mice. We found that unlike adult mice (14), naive T cells from neonatal BALB/c mice (1 day of age) did not respond to any of the self I-A^d peptides. However by day 11, response of unprimed T cells was similar (in magnitude) to that observed in adult mice but there was no reactivity to self peptide I-$A\beta^d$ 62-78. On the other hand, T cells from old BALB/c mice proliferated in response to self I-$A\beta^d$ 62-78 peptide. A number of other strains of mice showed a similar reactivity pattern to self I-Aβ 62-78 peptide. These results suggest that an immunodominant region of self MHC class II molecule does have an impact in limiting the T cell repertoire by being tolerogenic. These results are discussed in the context of breakdown of self tolerance with age in older mice.

MATERIALS AND METHODS

Mice CBA/j mice were obtained from Jackson laboratory (Bar Harbor, ME) and were 7-10 weeks of age. BALB/c, F1(BALB/c X CBA/j) and NOD mice were bred in HSLAS facility, MSB, University of Alberta, Edmonton. For neonatal mice, BALB/c mice were mated in HSLAS facility (MSB, U of A) and checked twice every day for birth of the neonates.

Antigens Ag used for this study included peptide sequences of I-$A\beta^d$, I-$A\alpha^d$, I-$A\beta^k$ and I-$A\beta^{NOD}$ (Table 3.1). All these peptide sequences were synthesized in this laboratory as previously described (14), using the Merrifield solid phase peptide synthesis technique on a Beckman 990C peptide synthesizer (Beckman

Instruments Inc., Fullerton, CA). The crude peptides were purified by reverse phase HPLC on a semipreparative synchropak RP-P C18(250x10mm ID) column using a linear gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile (1% of the second solvent/min). The composition of the peptide was confirmed by amino acid analysis using Beckman system 6300 amino acid analyzer. All peptides gave acceptable ratios of various amino acids. For functional assays, peptides were dissolved in saline by adjusting pH with 0.1 NaOH and were sterilized by filtration through a 0.22μm filter.

Purified protein derivative of tuberculin (Statens Serum Institute, Copenhagen, Denmark) and ConA (Calbiochem-Behering Corp., Lajolla, CA) were used in cultures as the positive controls in primed and unprimed T cell responses, respectively.

Medium RPMI 1640 medium (GIBCO Laboratories, Grand island, NY) containing $5x10^{-5}$M 2-ME, 10mM HEPES, 2mM glutamine, 5.0 IU/ml penicillin streptomycin (GIBCO) was used with 10% heat inactivated Fetal Bovine Serum (FBS) (BDH Inc., Edmonton, Canada).

Immunization Protocol For primed T cell proliferation assay, 8 to 10 weeks old mice were immunized subcutaneously with 25μg of peptide in 25μl saline mixed with equal volume of CFA in each hind foot pad. After 8-9 days, mice were sacrificed by CO_2 and draining lymph nodes were collected.

Isolation and culture of cells T cells were enriched from primed lymph nodes or unprimed spleen cells by nylon wool columns as

described earlier (18). For experiments with neonatal BALB/c animals, spleens from siblings of a litter were pooled. Briefly, $1x10^8$ lymph node or spleen cells in RPMI and 10% FBS in 1.0 ml of volume were loaded on to a 10 ml column containing 0.6g of nylon wool (Robbins scientific, Sunnyvale, CA) and incubated for 45 min at 37^0C in 10% CO_2, after which time effluent was collected. T cells ($5x10^5$) thus separated were cultured for 5 days in RPMI medium supplemented with $5x10^{-5}M$ 2-ME, 10mM HEPES, 2mM glutamine, 5.0 IU/ml penicillin streptomycin and 10% heat inactivated (FBS) in LINBRO 96 well flat bottomed plates. Irradiated (4000 rads) spleen cells (10^6 cells/well) from normal syngeneic mice (8-9 weeks old) were used as a source of APCs. I-A peptides were used at concentrations of 0.125μM to 125.0μM in various experiments. On 4th day of incubation, 1.0μCi of [^{3}H] TdR (NEN, Boston, MA) per well was added for 12-18 hr. Cells were then harvested on Titretak Harvester filters (Flow Laboratories, Mclean, VA). The filters were dried and counted on LKB Beta Liquid Scintillation Counter (LKB Instruments, Gaithsburg, MD).

RESULTS

1. Response of unprimed T cells from neonatal BALB/c mice towards self I-A^d peptides

In our earlier studies (14) we have reported that unimmunized syngeneic T cells from adult BALB/c mice proliferate in response to self I-A^d peptide which suggest that these cells are endogenously primed by self I-A^d peptides presented by self APCs

in vivo. In order to investigate whether T cells encounter self I-A peptides presented by self APCs *in vivo* to become reactive or tolerant, we examined the ontogeny of T cell proliferative responses to self I-A peptides. T cells from spleens of unprimed BALB/c mice of different ages (from day 1 of age) were separated by nylon wool column and cultured with irradiated spleen cells from adult BALB/c mice (8 weeks old) as APCs and various I-A^d peptides as antigens. The results presented in fig. 3.1 shows that T cells isolated from 1 and 3 day old BALB/c mice do not respond to syngeneic I-A^d peptides or allogeneic I-A^k peptide, however, the response to a mitogenic stimulus (ConA) suggests that these T cells are functional. T cells from 7 day old mice responded to ConA in the same fashion, there was some response to self peptides I-Aβ^d 1-14, 9-20 and I-Aα^d 1-17. The self peptide I-Aβ^d 62-78 and allo peptide I-Aβ^k 1-14 were not stimulatory. Furthermore, the T cells isolated from 11 day old BALB/c mice showed proliferative responses similar to that of the T cells from adult mice in magnitude. There was little response to the self peptide I-Aβ^d 62-78 and the allo peptide I-Aβ^k 1-14. An unrelated synthetic peptide EYK(EYA)4, known to have affinity for I-A^d and immunogenicity in H-2^d mice (19) did not stimulate proliferative responses in T cells isolated from unprimed BALB/c mice of various ages (fig. legend 3.1).

2. T cells from old mice respond to self I-Aβ 62-78 peptide

It was of interest to examine the T cell reactivity or tolerance to nonstimulatory peptide of I-Aβ chain (ie. I-Aβ 62-78) and therefore we determined the response of primed or unprimed T

cells from H-2^d, H-2^k, H-2^{dxk}, H-2^{NOD} strains of mice towards self I-Aβ 62-78 peptides. There was no proliferative response in T cells isolated from unprimed young (~8 weeks) mice towards self peptide I-Aβ 62-78 of the respective haplotypes (fig. 3.2). To examine primed T cell responses, young mice were immunized in hind foot pads subcutaneously with peptide and CFA. 8-9 days later, draining lymph nodes were taken out and T cells were purified by nylon wool columns (described in Materials and Methods). Purified T cells were then cultured with irradiated spleen cells as APCs and self I-Aβ 62-78 peptides at various concentrations and T cell proliferation was measured by ^{3}H Tdr uptake. PPD and ConA served as positive control to demonstrate that T cells were competent (fig. legend 3.2). As can be seen that except in NOD mice, there was no response in primed T cells from young mice towards self I-Aβ 62-78 peptide (fig. 3.2). In contrast, T cells isolated from lymph nodes of mice primed with allo I-Aβ 62-78 peptides proliferated in response to the respective allo I-Aβ 62-78 peptide (Table. 3.2). To further determine if tolerance/unresponsiveness to self I-Aβ 62-78 is maintained throughout life, we examined the response of T cells from old mice (>6 months old) of different haplotypes towards their respective self I-Aβ 62-78 peptides. T cells were separated from spleen cells of unprimed old mice and cultured with irradiated spleen cells (from 8 wk old syngeneic mice) and respective self I-Aβ 62-78 peptide at various concentrations. Surprisingly, the T cell proliferative response to this self I-Aβ peptide was considerably greater than that of T cells from young mice in all of the haplotypes of mice tested. Overall magnitude of response was the highest in

autoimmune diabetic NOD mice (fig. 3.2). Therefore T cell tolerance to a self peptide corresponding to an immunodominant region on I-A seems to be some how broken at old age. The mechanism of tolerance induction and breakdown is yet unclear.

DISCUSSION

The process of thymic selection during T cell ontogeny ensures the deletion/inactivation of potentially autoreactive T lymphocytes (1, 2, 21), therefore it is generally expected that all potential self determinants, ie. self peptides that bind to MHC molecules with high affinity would be nonimmunogenic due to clonal deletion or inactivation of corresponding T cells. However, using a foreign antigen system it has been shown that T cells specific for minor or cryptic determinants can escape tolerance induction (22). This evasion is apparently based on ineffective *in vivo* processing, and failure to assemble enough complexes of the minor determinants and MHC molecules. We and others have recently reported (14, 16) the presence of self MHC peptide reactive T cells in syngeneic mice. Our results further suggested that syngeneic T cells can be primed by self MHC peptides presented by APC *in vivo* in naive mice. In this report our results demonstrate that early in ontogeny, developing T cells encounter the epitopes of self MHC class II molecules presented by APC and get primed. However, it remains unclear whether T cells encounter the peptides of MHC class II molecules or the region of MHC molecules in intact molecule. The former possibility is suggested by the observation that peptides corresponding to the

region of MHC molecule which forms the floor of the putative ag binding groove (ie. amino terminal hypervariable region) (18), also seems to prime the T cells *in vivo*.

The fact that the peptide corresponding to the region I-Aβ 62-78 does not stimulate proliferation of syngeneic T cells, is significant (as discussed earlier). However, a considerable T cell proliferative response to self I-Aβ 62-78 peptide was found in T lymphocytes isolated from old mice (>6 months old). The apparent breakdown of tolerance to an immunodominant region of MHC II molecules could be speculated on the basis of various possibilities. One possibility is that this immunodominant region of self MHC class II molecule include a determinant of profound importance in the acquisition of repertoire and is strongly regulated by regulatory cells. Lack or reduction of these regulatory cells in old age may activate the self MHC class II peptide I-Aβ 62-78 reactive T cells.

Second, mimicry between a pathogen and the third hypervariable region of β chain of a class II molecule (23) can result in expansion of self reactive clones during an infection and tolerance being broken due to expansion of autoreactive cells. Alternatively, the pathogen may break tolerance in potentially autoreactive T cells by reversing the anergy by activation of alternative reaction pathways (24).

Third, efficient processing and presentation of self I-Aβ 62-78 peptide during early life may have played a role in maintaining nonresponsive status of T cells, which could have been replaced by insufficient presentation of self I-Aβ 62-78 peptide in

old mice, thus reversing the nonresponsiveness of anergic T cells (25).

Finally, an extrathymic pathway of T cell differentiation in mice has been suggested (26) to be activated as a function of age, which leads to age dependent increase of intermediate TCR cells in liver and periphery. These intermediate TCR cells may be responsible for the observed reactivity to the self I-Aβ 62-78 peptide and may eventually lead to autoimmune like condition.

Although, the mechanism of induction and subsequent breakdown of tolerance to a self I-Aβ 62-78 peptide (corresponding to an immunodominant region on I-A molecule) is not evident from our studies, our result suggests incomplete tolerance to self components. These potentially autoreactive T cells may become involved in autoimmune diseases. The T cell proliferative response to self I-Aβ 62-78 peptide is higher in diabetic NOD mice as compared to H-2^k, H-2^d and H-2^{dxk} strains of mice with no known autoimmune disease. The relevance of T cell response to self I-Aβ 62-78 peptide in autoimmune condition is not direct but can be related because autoimmune diabetes is a multigene and multifactor dependent autoimmune disease (27).

In conclusion, the incomplete tolerance to self components may not be a cause of great concern, unless under the condition of pathogenesis or combination of factors (ie. genetic and environmental) where self reactive T cells may play a role in autoimmune disease. In spite of this potential, the immune system has evolved to not eliminate all self reactive T cells and positively influence the T cell repertoire.

Table 3.I

Various synthetic peptides used in this study

Synthetic Peptide	Region on intact I-A molecule	Sequence of peptide
I-Aβ^d	β1 domain	
1-14	HV1	GNSERHFVVQFKGE
9-20	HV1	VQFKGECYYTNG
62-78	HV2	NSQPEILERTREVDTA
I-Aα^d	α1 domain	
1-17	HV1	EDDIEADHVGFYGTTVY
I-Aβ^k	β1 domain	
1-14	HV1	GNSERHFVHQFQPF
62-78	HV2	NKQ YL ERTRAELDTA
I-Aβ^{NOD}	β1 domain	
62-78	HV2	NKQ YL ERTRAELDTA

Table 3.2

Response of Young mice to allo I-Aβ 62-78 peptide

Strain of mice[a]	Priming Ag[b]	Ag in culture	Conc. of Ag in culture (μM)	[3H] TdR incorporation CPM ± S. D.
BALB/c	I-Aβ^k 62-78	I-Aβ^k 62-78	0.0	4455 ± 2932
			1.25	20201 ± 1362
			12.5	33599 ± 2937
			125.0	40438 ± 3952
CBA/j	I-Aβ^d 62-78	I-Aβ^d 62-78	0.0	955 ± 190
			1.25	4981 ± 940
			12.5	14905 ± 3617
			125.0	5844 ± 613
NOD	I-Aβ^d 62-78	I-Aβ^d 62-78	0.0	1936 ± 378
			1.25	13683 ± 1557
			12.5	28176 ± 3262
			125.0	19474 ± 5459

a. Mice were 8-9 weeks old.

b. mice were immunized with Ag (25μg) in CFA in both hind foot pads.

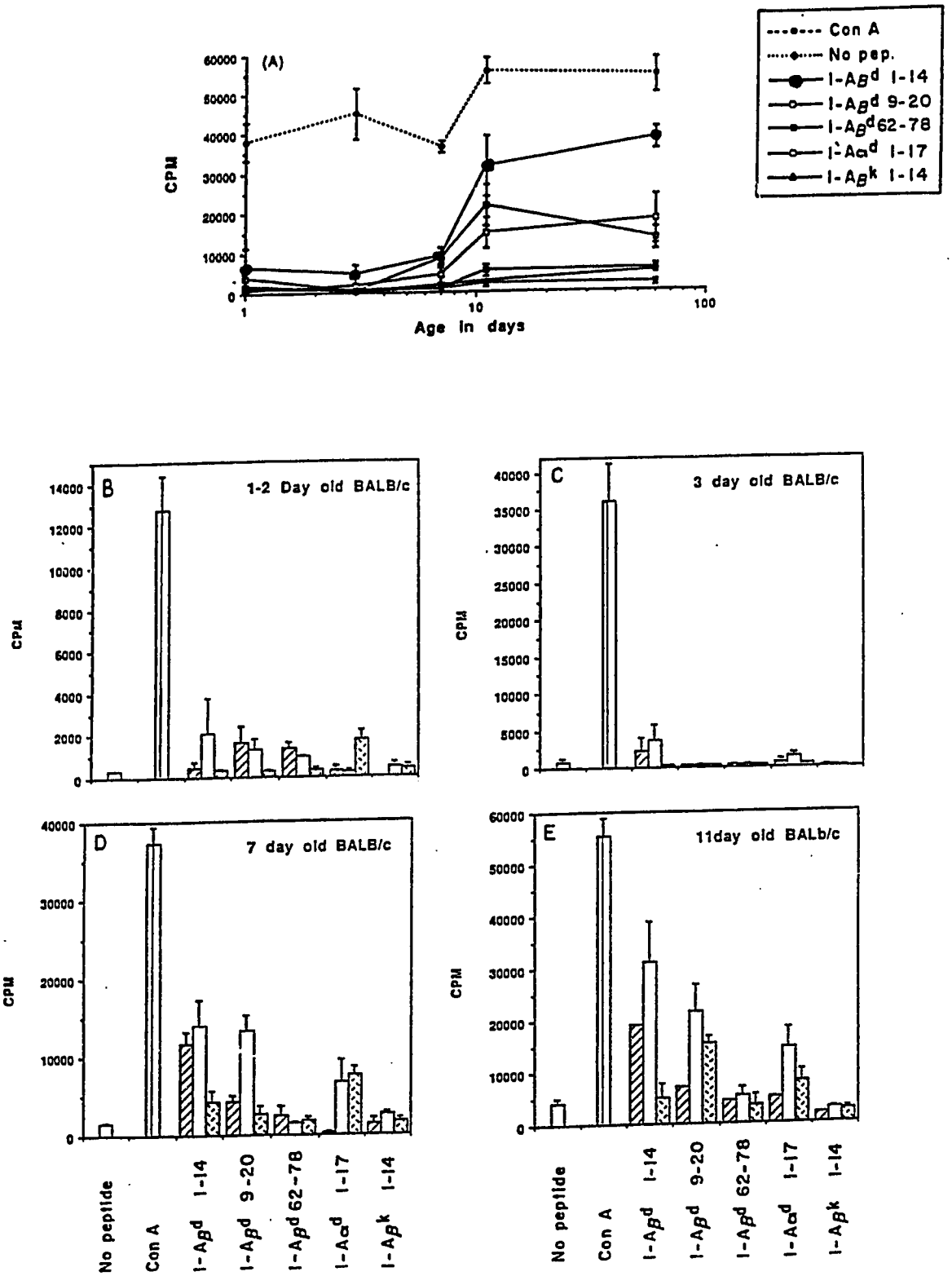
(A)
CPM
Age in days
Con A
No pep.
I-Aβd 1-14
I-Aβd 9-20
I-Aβd62-78
I-Aαd 1-17
I-Aβk 1-14
B
1-2 Day old BALB/c
C
3 day old BALB/c
D
7 day old BALB/c
E
11day old BALb/c
No peptide
Con A
I-Aβd 1-14
I-Aβd 9-20
I-Aβd 62-78
I-Aαd 1-17
I-Aβk 1-14

Fig. 3.1 ***Response of unprimed T cells from neonatal BALB/c mice towards I-A peptides.***

A. Summary of (B-E) proliferative responses of T cells from BALB/c at different ages. I-A peptides were used at a concentration of 12.5 μM. B-E. Proliferative response of unprimed T cells separated from 1-2 day, 3 day, 7 day, 11 day old BALB/c mice respectively. I-A peptides were used at 1.25μM (▨), 12.5 μM (❑), and 125.0 μM (▩) concentration. ConA was used at a concentration of 0.2 μg/well.

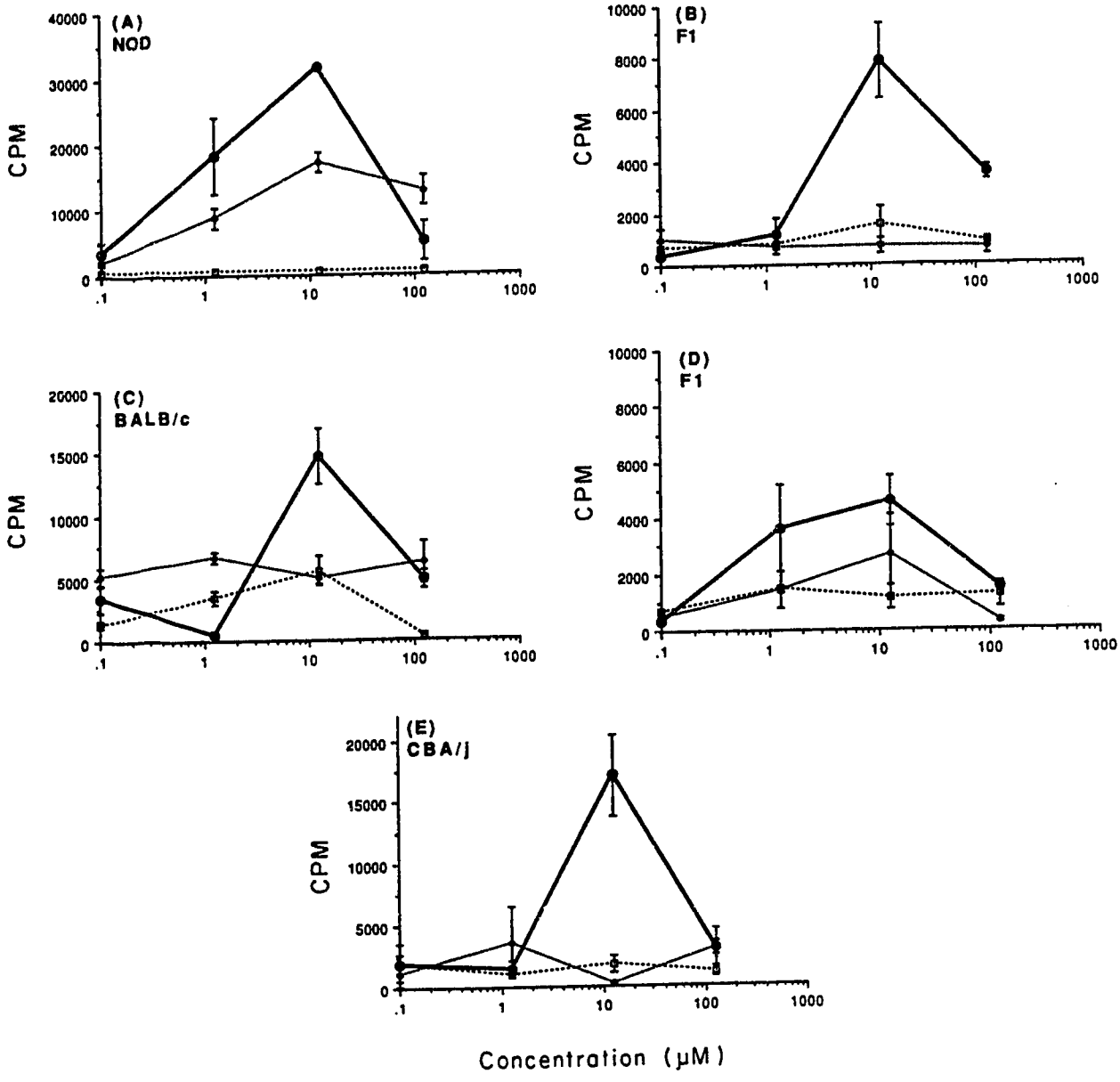
(A)
NOD
(B)
F1
(C)
BALB/c
(D)
F1
(E)
CBA/J
CPM
Concentration (μM)

Fig. 3.2 ***T cell proliferative response towards self I-Aβ 62-78 peptide.***

Responses are shown for young (8 week old) primed (·····●·····) young unprimed (--□---), and old (> 6 months old) unprimed (—●—) mice. (A) response of NOD mice towards I-$A\beta^{NOD}$ 62-78 peptide, (B) response of F1 (BALB/cXCBA/j) towards I-$A\beta^{d}$ 62-78 peptide, (C) response of BALB/c to I-$A\beta^{d}$ 62-78 peptide, (D) response of F1 to I-$A\beta^{k}$ 62-78 peptide and (E) response of CBA/j mice to I-$A\beta^{k}$ 62-78 peptide. Primed and unprimed mice are as defined in Materials and Methods.

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CHAPTER IV

IMMUNOGENIC PEPTIDES INCREASE CELL SURFACE EXPRESSION OF MHC CLASS II MOLECULES

INTRODUCTION

Protein antigens are taken up by antigen-presenting cells and processed into peptides that bind to MHC Class II molecules for presentation to helper T cells. APC can not distinguish between self and nonself proteins and it is likely that self peptides are continuously generated and presented in association with MHC Class II molecules. Both self and nonself peptides have been found to constitutively occupy the antigen binding groove of Class II molecules (1-6). The antigen specificity of T cells directed to a given antigen is critically dependent upon the nature of the peptide-MHC complexes thus generated.

During the intracellular transport of nascent MHC Class II $\alpha\beta$ complex, transit through the post Golgi compartment is intersected by the endocytic pathway (7, 8), where specific proteolytic cleavage and subsequent dissociation of Ii chain (9) is presumed to be followed by charging of MHC Class II molecule with degraded peptide antigen. MHC Class II molecules, recycling from the surface to the interior of APC, also interact with processed antigen in the endosome by peptide exchange (10, 11). Even with

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the detailed understanding of antigen processing and presentation events, the relationship of antigen processing to synthesis, assembly, transport and expression of MHC molecules has not been explored. Some experiments using purified MHC Class II molecules have suggested that stable binding of peptides is necessary for Class II dimers to enter into a compact state and be transported to the cell surface (12). Similar results have come from studies using cell lines defective in Class I molecule assembly, demonstrating a critical role of peptides in stabilizing MHC Class I heavy chain-β_2m complex and in facilitating their movement to the cell surface (13-17).

We have investigated the effect of peptide ligands on the cell surface expression of MHC Class II I-A^d molecules in a B cell hybridoma (TA3). In our preliminary experiment it was observed that after *in vitro* culturing, constitutive cell surface expression of I-A^d on these cells decreased considerably. Addition of I-A restricted exogenous antigens including a self I-Aβ^d amino terminal peptide fragment, which we recently found to bind intact I-A^d on the cell surface with high affinity (19), significantly (>100% or >two fold) increased the cell surface expression of I-A^d on TA3 cells. Addition of I-A^k restricted exogenous antigens had no effect on the expression of I-A^d on these cells. These results suggest that the decrease in surface expression of I-A^d on TA3 cells may have resulted from its failure to be saturated with endogenous self peptide ligands. These ligands would include peptides derived from self MHC Class II molecules.

MATERIALS AND METHODS

Antigens: Antigens used for this study included peptide sequences of I-A chain from the amino terminus, and a synthetic polypeptide antigen Poly EYK(EYA)$_5$ (Poly18) and its peptide fragments. All these peptides were synthesized in this laboratory according to previously reported procedures (18, 19). Hen egg lysozyme (HEL) was purchased from the Sigma Chemical Company, St. Louis, MO.

Cells and Hybridomas: TA3 cells were obtained from Dr. L. Glimcher, Harvard Medical School, Boston. These are surface IgM$^+$, I-A^{d,k} and I-E^{d,k} expressing B cell hybridoma derived from the fusion of lipopolysaccharride stimulated (BALB/c x A/J)F1 spleen cells with the BALB/c (H-2^d) B cell lymphoma (20). Poly 18 reactive I-A^d restricted T cell hybridoma A.1.1 was generated in this laboratory (18) and is readily activated by a synthetic peptide (K4) when presented by fixed or unfixed APCs. An IL-2 dependent T cell line CTL-L was used to assay IL-2 content as previously described (Chapter II).

Culture Medium: RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 5 x 10^{-5}M 2-ME, 10 mM HEPES, 2 mM glutamine, 5.0 1U/ml penicillin streptomycin (GIBCO) was used with 10% heat inactivated Fetal Bovine Serum (FBS) (BDH Inc., Edmonton, CANADA).

Monoclonal Antibody: Hybridoma cells producing the anti I-A^d monoclonal antibody MKD6 were purchased from American Type

Culture Collection, Rockville, MD. MKD6 antibody used in these experiments was used as ascites (18).

Antigen Presentation Assay: Fixed or unfixed TA3 cells (5 x 10^4) were incubated with 10^5 T cell hybridomas A.1.1 with or without (5-250 μg/ml) K4 peptide antigen in a total volume of 200 μl of culture medium in flat bottomed 96-well microtitre plates. Fixation of TA3 cells by glutaraldehyde was carried out as previously described (19). After 24 hours, supernatant was collected and assayed for IL-2 content in a secondary culture by using IL-2 dependent T cell line CTL-L cells. CTL-L (10^4) cells were cultured for two days with serial dilutions of primary supernatant (starting at 12.5% primary supernatant), and the degree of stimulation was measured by the incorporation of (1 μCi/well) ^{3}H-Thymidine (New England Nuclear).

Immunofluorescence Analysis: TA3 cells (1 x 10^6) were incubated with 100 ml of appropriately diluted (1:100) quantity of MKD6 (ascites) for 20 minutes at 0°C. Cells were washed with washing buffer (PBS containing 2% FBS and 0.5% NaN_3), stained by treatment with 100 μl of fluorescein conjugated goat anti-mouse Ig (Fab Fragments only, TAGO Inc., Burlingame, CA, USA) for 20 minutes and washed again with washing buffer. The cells were then fixed using 0.5 ml of PBS containing 2% Formalin. Prior to staining for FACS analysis, 10^6 TA3 cells were incubated with or without the desired concentration of peptides in 400 μl in RPMI 1640 culture medium for varying lengths of time and washed in washing buffer. For B220 expression, MAb against B220 (mouse) was used as supernatant (salt

cut) obtained from culturing RA3.3A1 hybridoma cells obtained from ATCC, Rockville, MD. The second antibody used was FITC conjugated goat anti rat Ig (Fab Fragment, TAGO Inc., Burlingame, CA, USA).

Fluorescence profiles generated by MAb binding to TA3 cells was analysed on a FACScan (Becton-Dickinson) in which a laser was used to excite FITC. Files were collected for 5,000 cells from each sample, and measurements of side scatter (as a measure of granularity) and forward scatter (as a measure of size of the cells) were stored along with fluorescence profiles. Data was plotted as the number of cells (ordinate) versus the log fluorescence intensity. All fluorescence profiles generated by antibody binding to TA3 were symmetrical curves. The mean log fluorescence intensity of staining was $\simeq$ 15 channel units when TA3 cells were treated with only the second step antibody, Fluorescein (FITC) conjugated goat anti mouse Ig. This intensity was always lower than that observed when TA3 cells were treated first with anti I-A^d antibody and then with second step FITC conjugated goat anti mouse Ig, even in the TA3 cells expressing the lowest density of I-A^d molecules on the surface. This suggested that the level of expression of surface IgM molecules on TA3 cells did not change with a change in I-A expression (Figure 4.1a). Percent of initial level of I-A^d for each experiment was calculated as:

$$\frac{\text{(channels of mean fluorescence intensity of cells incubated with peptide)}}{\text{(channels of mean fluorescence intensity of cells incubated without peptide)}} \times 100\%$$

Channels of the background fluorescence intensity observed in the absence of the first Ab (ie. in the presence of FITC conjugated

second Ab only) was mathematically subtracted from each sample before calculating the percent of initial level of I-A^d in presence or absence of immunogenic peptides.

RESULTS

1. Decreased Cell Surface Expression of I-A^d on TA3 Cells in Culture

TA3 cells, cultured for various time periods were examined for the cell surface expression of I-A^d. Surprisingly, after 8 weeks in culture, the density of I-A^d surface expression decreased to approximately 66% (Figure 4.1a). By 20 weeks, the expression of I-A^d was less than 25% of the level expressed on the original TA3 cells (Figure 4.1a). No further decrease in I-A^d expression was observed after this time period, and the surface expression never reached below a level representing 20 channels of mean fluorescence intensity.

Fluorescence profiles generated by the binding of a mAb directed against B220 molecules (B cell marker) suggested that the level of B220 expression on the high and low I-A^d expressing TA3 cells was almost equivalent (Figure 4.1b). Level of expression of other I region molecules e.g. I-A^k and I-$E^{d,k}$ was similar on the low and high I-A^d expressing TA3 cells as determined by binding to MAb directed against I-A^k and I-$E^{d,k}$ (Fig. 4.1b). Therefore, low I-A^d expressing TA3 cells have a defect in I-A^d expression and molecules unrelated to I-A^d did not seem to be affected.

2. Effect of Immunogenic Self I-A Peptides on the Expression of I-A^d on TA3 Cells

To determine if the insufficient peptide ligands were limiting the expression of I-A^d on TA3 cells, we exposed low I-A^d expressing TA3 cells to the I-Aβ^d1-14 amino terminal peptide which we have previously shown to have high affinity to intact I-A^d molecule and high immunogenicity in H-2^d mice (19). Incubation of low I-A^d expressing TA3 cells with IAβ^d1-14 peptide increased surface expression of I-A^d to more than 200% of the level on cells cultured without additional peptide (Figure 4.2). As a control we tested other amino terminal peptides I-Aα^d1-17 and I-Aβ^k1-14 . These peptides had very little or no effect on the expression of I-A^d molecules on the low I-A^d expressing TA3 cells at all the concentrations tested (Figure 4.3). These peptides have lower affinity for intact I-A^d and lower immunogenicity in H-2^d mice as compared to the peptide I-Aβ^d 1-14 (19). These results implied that only the peptides having high affinity to I-A^d induce the increase in its expression. We also examined the effect of I-Aβ^d peptides on high I-A^d expressing TA3 cells. Exposing high I-A^d expressing TA3 to a number of self I-Aβ^d peptides revealed almost no difference in I-A^d expression on these cells than the cultures without an exogenous peptide (Table 4.1).

3. Time Course of the Induction of Increase in Cell Surface Expression of I-A^d by I-Aβ^d1-14 Peptide

We incubated the low I-A^d expressing TA3 cells to various concentrations of I-Aβ^d 1-14 peptide for varying lengths of

time (Figure 4.4). Culturing the cells with peptide for 10 minutes had almost no effect at any concentration tested. However, the effect could be detected at 2 hours and reached its maximum in 24 hours. After 24 hours of incubation of TA3 cells in presence of I-Aβ^d 1-14 peptide, cell surface expression of I-A^d was increased to more than 200% of the level on cells cultured without the peptide. The expression of I-A^d is restored in less than 24 hours in contrast to its loss which occurs over 8-20 weeks.

4. I-A^d Restricted Exogenous Peptide Antigen Poly 18 Increases the Expression of I-A^d on TA3 Cells

To extend the observation of the effect of self peptides on I-A^d expression to the range of foreign peptide antigen, I-A^d and I-A^k restricted foreign antigen were used. The response to Poly 18 is I-A^d restricted (18), suggesting its ability to bind to I-A^d molecules. We have previously shown that Poly 18 derived peptide antigens bind to I-A^d (21). We cultured low I-A^d expressing TA3 cells with Poly 18 (250 μg/ml) for 24 hours and examined the expression of I-A^d on TA3 cells cultured in the presence or absence of antigen (Figure 4.5). Surface expression of I-A^d on TA3 cells cultured with Poly 18 was greater than 200% of the level on cells cultured without antigen. At the same time, culturing an I-A^k restricted foreign antigen HEL did not have any effect on the expression of I-A^d. Incubation of CBA/J (H-2^k) spleen cells with HEL has been reported to increase the surface expression of I-A^k (5).

5. Presentation of Antigen by TA3 Cells Expressing High and Low Levels of I-A^d on the Surface

We determined the ability of low and high I-A^d expressing TA3 cells to present I-A^d restricted Poly 18 derived synthetic peptide EYK(EYA)$_4$ [K4] to a Poly 18 specific T cell hybridoma A.1.1 (18). No significant difference was observed in the stimulation of A.1.1 T cell [at 50 μg/well of K4] at high concentration of antigen when either high or low I-A^d expressing TA3 cells were used as APCs (Figure 4.6). It is likely that K4 has high affinity for I-A^d and generally <1% of I-A molecules on the cell surface are sufficient to present antigen for T cell stimulation (22). On the other hand, K4 may have induced increased expression of I-A^d on the cell surface, hence compensating for the ability to present antigen. The later possibility was further tested by using glutaraldehyde fixed TA3 cells (low and high I-A^d expressing) as APCs. Glutaraldehyde inhibits the cellular exchange with the environment by cross linking surface molecules, thus will prevent the induction of I-A^d expression by peptide antigen. Low I-A^d expressing fixed TA3 cells stimulated A.1.1 T cells to a lower degree as compared to high I-A^d expressing fixed TA3 cells [at 50 μg/well of K4] (Figure 4.6). These results further suggest that the peptide antigens upregulated the expression of I-A^d on lower I-A^d expressing unfixed TA3 cells thus boosting their antigen presentation capacity.

DISCUSSION

TA3 cells in culture appear to lose the cell surface expression of I-A^d, although a constitutively low level expression of I-A^d remains. However, expression of a B cell surface marker (B220

molecules) on these cells does not seem to change with time. One possible explanation for these results is that an insufficient amount of certain endogenous self peptides may be generated in these cells which limits the expression of I-A^d. In normal high I-A^d expressing cells, endogenous self peptides may be playing a role in stabilization and transport of I-A^d. Such peptides may be necessary for binding to the antigen binding groove of I-A^d to enhance the stability of $\alpha\beta$ chain pairing which is crucial for the surface expression of Class II MHC molecules (12). Our results show that the low level expression of I-A^d can be upregulated to as much as 200% of initial by exposing TA3 cells to certain peptides with high affinity for I-A^d. In our hands the peptide I-$A\beta^d$ 1-14 seems to be the most efficient among the three amino terminal peptides of I-A tested in increasing I-A^d surface expression in low I-A^d expressing TA3 cells, which could be due to its high affinity with the intact I-A^d molecule (19) and moreover, it may represent an endogenous self peptide. This hypothesis is further supported by the fact that an I-Eα derived peptide could be eluted from the antigen binding groove of I-A^b molecule (23). It can be implied that self I-Eα peptide may help in regulating the expression of I-A^b molecules physiologically.

Another interesting aspect of the study was that it took 8-20 weeks for the loss of I-A^d expression on TA3 cells. However, adding peptides such as I-$A\beta^d$ 1-14 restored the expression of I-A^d within less than 24 hours. This may have physiological significance in antigen presentation by the APC. However, the increase in surface expression of I-A^d in TA3 seems to be peptide dependent, but not peptide specific because a synthetic polypeptide antigen Poly 18

which has a high affinity to I-A^d but does not represent an endogenous peptide has a similar effect. This may be more related to the affinity of the peptide for I-A^d. Examining the dose of I-$A\beta^d$ 1-14 peptide required to increase the expression of I-A^d on TA3 cells, concentration of peptides as low as 1.25 μM was also effective, whereas in the case of I-A^k expression, a minimum concentration of 2 mg/ml (144 μM) of HEL was required to induce the increase in I-A^k expression (5). The difference could be reasoned on the basis of processing requirements of these antigens for presentation to T cells. I-$A\beta^d$1-14 or Poly 18 antigen are small antigen peptides and do not need processing (19, 21), whereas HEL is a large molecule and needs processing before presentation to T cells (24). We have not yet identified the mechanism by which these antigenic peptides can increase I-A^d surface expression. A number of possibilities exist.

First, in an intracellular compartment of low pH, separate α and β chains bind to peptides and are transported to the cell surface individually and assembly takes place at the cell surface (25). Second, peptides bind to the unstable form of MHC Class II dimer and induce the conversion to compact form leading to stabilization and surface expression (5, 12). Third, empty MHC Class II dimers are expressed on the cell surface and our MAb MKD6 is not able to recognize the I-A^d molecules unless they are bound to peptide. The time course of the effect of peptides on I-A expression however favor a mechanism which involves transport of intracellular pool of I-A to the cell surface. Since during intracellular trafficking from ER to cell surface, Class II molecules have a half time of 2-4

hours for surface expression and the effect of I-Aβ^{d}1-14 peptide on I-A^d expression was not much apparent before 2 hours.

Investigation of antigen presenting capacity of high or low I-A^d expressing TA3 cells to I-A^d restricted Poly 18 reactive T cell hybridoma revealed almost no difference, which can be explained by the fact that less than 1% of Class II molecules are involved in presenting a particular antigen and also the phenomenon that peptides with high affinity to Class II molecules can increase the expression of Class II molecules hence manipulating their antigen presenting ability. The latter possibility is supported by the antigen presentation experiment using glutaraldehyde fixed TA3 cells on APCs.

An obvious question about the role of fetal bovine serum (FBS) derived peptides, can be explained on the basis of low affinity of these peptides to I-A^d molecules. Among the three of our closely related I-A peptides (all are amino terminal peptides of I-A α and β chains), only I-Aβ^d 1-14 peptide has an effect of increasing I-A^d expression on the cell surface, which has been shown to have the highest affinity to intact I-A^d among these three peptides. The requirement of high affinity of antigen to intact Class II molecule for the induction of Class II heterodimer expression suggests a need of stringent interaction between peptide and Class II chains. Other peptides for Class II molecules served as good internal controls in these experiments. Similar kinds of high affinity requirements has previously been reported for Class I MHC expression (13). Since MHC molecules serve similar functions, it is not unexpected that they

might have similar requirement for antigen-mediated upregulation of their expression on the cell surface.

In a parallel antigen presenting system, peptides have been found to stabilize the conformation of ligand binding region (α_1/α_2) of Class I molecules and association of Class I heavy chain to β_2m leading to the induction of the expression of Class I molecules (13). Results from experiments with purified native Class I proteins (26) have further shown that Class I antigen complex is most stable and efficient when both antigenic peptide and β_2m are incubated simultaneously with immobilized Class I heavy chain. On the other hand, sequential incubation of immobilized Class I heavy chain with β_2m followed by peptide results in less efficient Class I-antigen complex formation, possibly due to instability of the empty Class I molecules. In contrast to MHC class I molecules, it has been reported (27) that MHC class II heterodimers can remain associated in the absence of antigenic peptides. Empty MHC class II molecules can also be expressed on the cell surface. Peptide binding to MHC class II heterodimer has been found (5, 27) to stabilize the association. The differences in the role of antigenic peptide in stability class II vs association of class I heterodimers obviously explains our observations that less dramatic effects of peptides can be seen on class II surface expression in comparison to class I expression.

In conclusion, our study suggests a crucial role for immunogenic peptides in regulating the surface expression of MHC Class II I-A^d molecules on antigen presenting cells. These results will be of value in understanding the activation of class II restricted T helper cells following antigen priming.

Table 4.1

Effect of I-A_β^d peptides on TA3 cells expressing high level of I-A^d

Peptides[a] (125 μM)	Mean Fluorescence Channel[b]
Background (no MKD6)	20
Control (no peptide)	90
I-A_β^d (1-14)	90
I-A_β^d (9-20)	90
I-A_β^d (21-36)	80
I-A_β^d (62-78)	90
I-A_β^d (84-91)	90

a. 10^6 TA3 cells were incubated for 24 hr in presence or absence of I-A peptides before staining for FACS.

b. Level of I-A^d on the TA3 cells is represented as the channels at which the mean fluorescence intensity occurred.

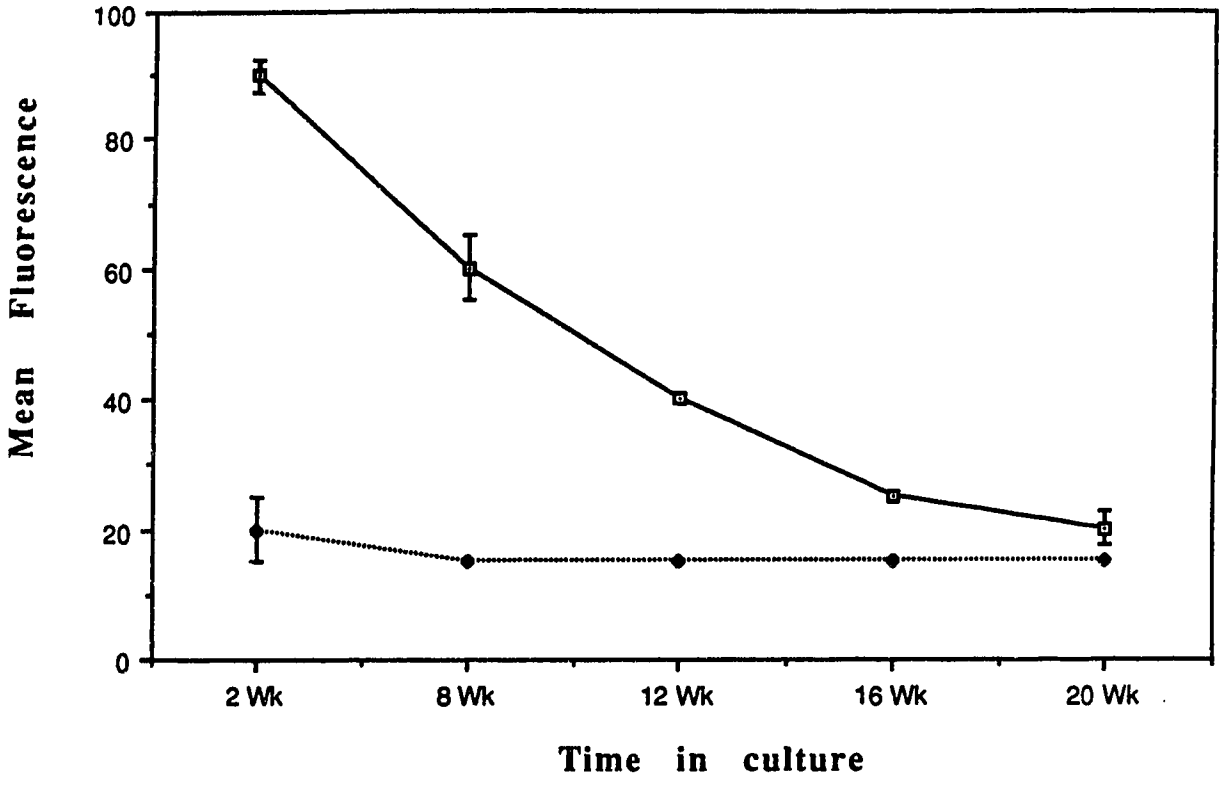

Figure 4.1a ***Time course of expression of I-A^d on TA3 cells in culture.***
TA3 cells were cultured in RPMI 1640 culture medium (as described in Materials and Methods) for 20 weeks and I-A^d expression was determined by immunofluorescence analysis at different times. Profile representing background (-----◆-----) staining were obtained using only the second antibody. I-A^d expression is represented by mAb MKD6 binding (—⊡——). The results were obtained as mean fluorescence in logarithmic scale and expressed in channels.

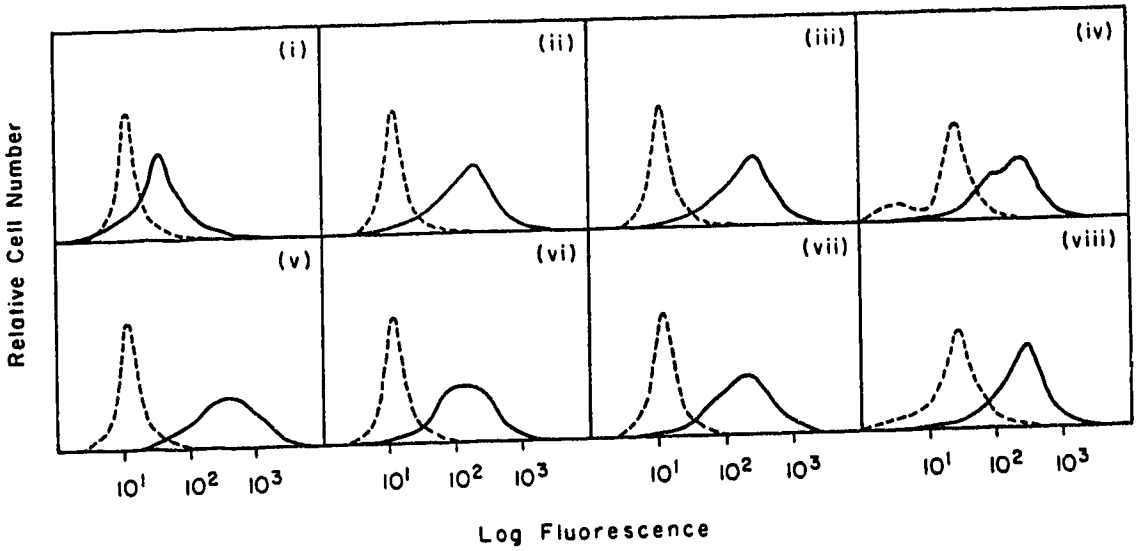

Figure 4.1b ***Expression of B220, I-A^k and I-E^{d,k} molecules on TA3 cells with high and low expression of I-A^d on the cell surface.***

Upper panel represents the low I-A^d expressing and lower panel represents high levels of I-A^d expressing TA3 cells. Dotted lines represent the staining with second antibody only, FITC conjugated GαM (i, ii, iii, v, vi and vii) and FITC conjugated GαRAT (iv and viii). The solid lines in profiles (i) and (v) represent I-A^d expression, (ii) and (vi) represent I-A^k expression, (iii) and (vii) represent I-E^{d,k} expression and (iv) and (viii) represent B220 expression.

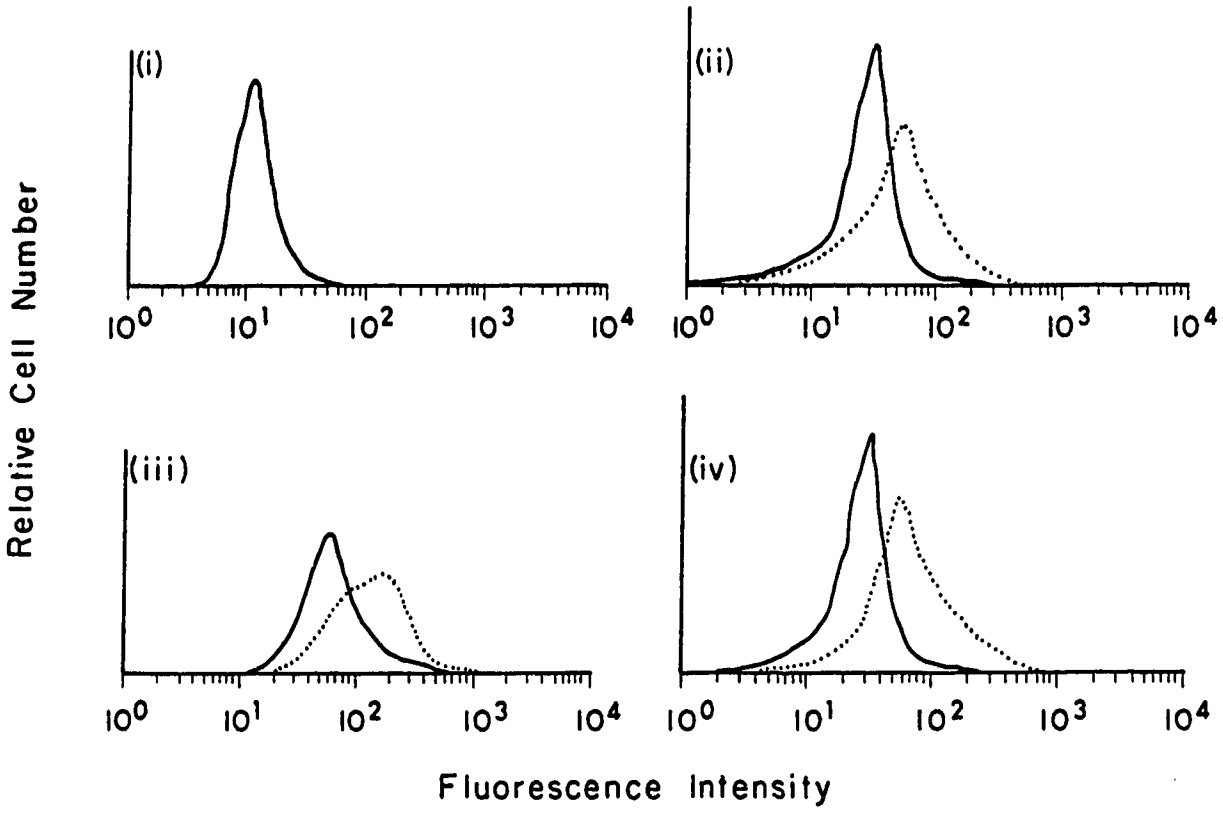

Figure 4.2 ***I-Aβ^d1-14 peptide increases surface I-A^d expression on low I-A^d expressing TA3 cells.***
10^6 TA3 cells were incubated with I-Aβ^d1-14 peptide (1.25 μM) for 24 hours and stained for FACS analysis as described in Materials and Methods. (i) FACS profile obtained with second antibody binding. (ii), (iii) and (iv) represent three independent experiments where solid and dotted lines represent the sample cultured in the absence and presence of peptide respectively.

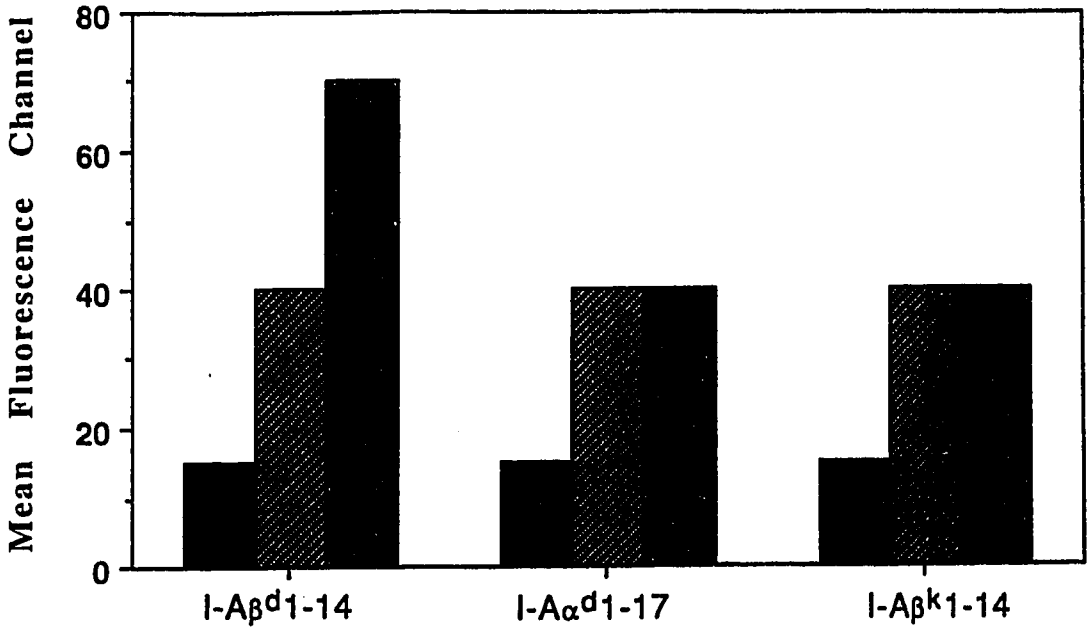

Figure 4.3 ***Effect of amino terminal I-A peptides on the expression of I-A^{d}.***

10^{6} TA3 cells expressing low levels of I-A^{d} were exposed to I-A peptides (viz. I-Aβ^{d}1-14, I-Aα^{d}1-17 and I-Aβ^{k}1-14) at 125 μM for 24 hours and examined for levels of I-A^{d} expression on the cell surface by FACS analysis. Results are expressed in channels (ordinate) at which mean fluorescence intensity was observed. Background fluorescence intensity (■) was obtained when TA3 cells were stained with only the second antibody. TA3 cells were incubated in the absence (▨) or presence of 125μM (■) of peptides before staining for FACS. The figure represents one experiment out of two repeated experiments.

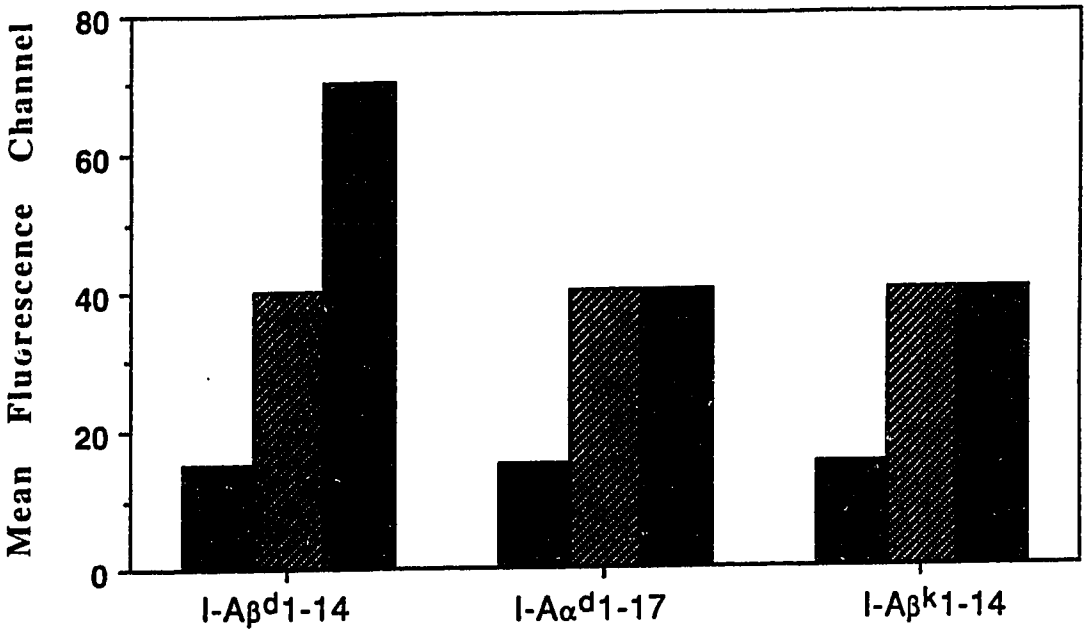

Figure 4.3 ***Effect of amino terminal I-A peptides on the expression of I-A^{d}.***

10^{6} TA3 cells expressing low levels of I-A^{d} were exposed to I-A peptides (viz. I-Aβ^{d}1-14, I-Aα^{d}1-17 and I-Aβ^{k}1-14) at 125 μM for 24 hours and examined for levels of I-A^{d} expression on the cell surface by FACS analysis. Results are expressed in channels (ordinate) at which mean fluorescence intensity was observed. Background fluorescence intensity (■) was obtained when TA3 cells were stained with only the second antibody. TA3 cells were incubated in the absence (▨) or presence of 125μM (■) of peptides before staining for FACS. The figure represents one experiment out of two repeated experiments.

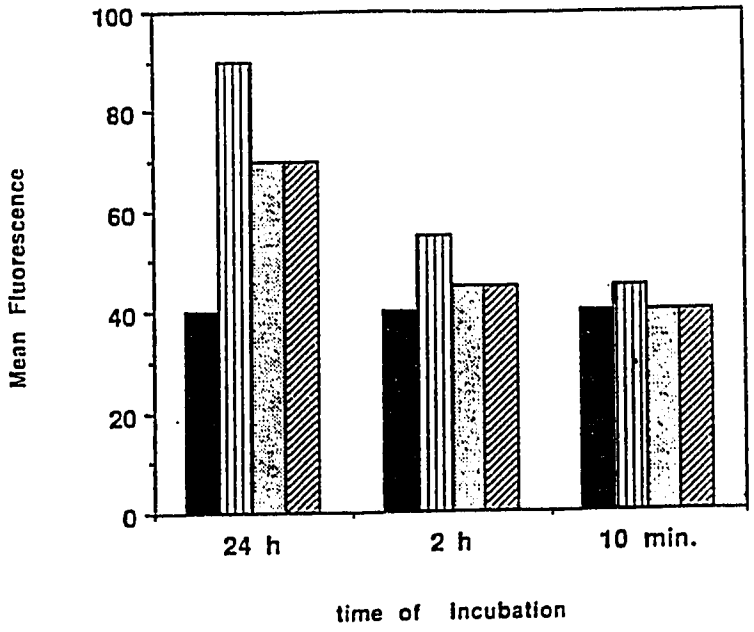

Figure 4.4 ***Time course of effect of I-Aβ^{d}1-14 peptide on the expression of I-A^d.***

Low levels of I-A^d expressing TA3 (10^6) cells were incubated with varying concentrations (1.25 μM to 125.0 μM) of I-Aβ^{d}1-14 peptide for varying lengths of time and examined for cell surface expression of I-A^d by FACS analysis. Mean fluorescence was represented by channel at which mean fluorescence intensity occured. Mean fluorescence of the sample without the first antibody (anti I-A^d) was considered as background and was observed at 15 channels. (■) represents the cells incubated without peptide whereas (▥), (▨) and (▨) represent the cells cultured with 125 μM, 12.5 μM and 1.25μM of I-Aβ^d 1-14 peptide respectively. The figure represents one out of three repeated experiments.

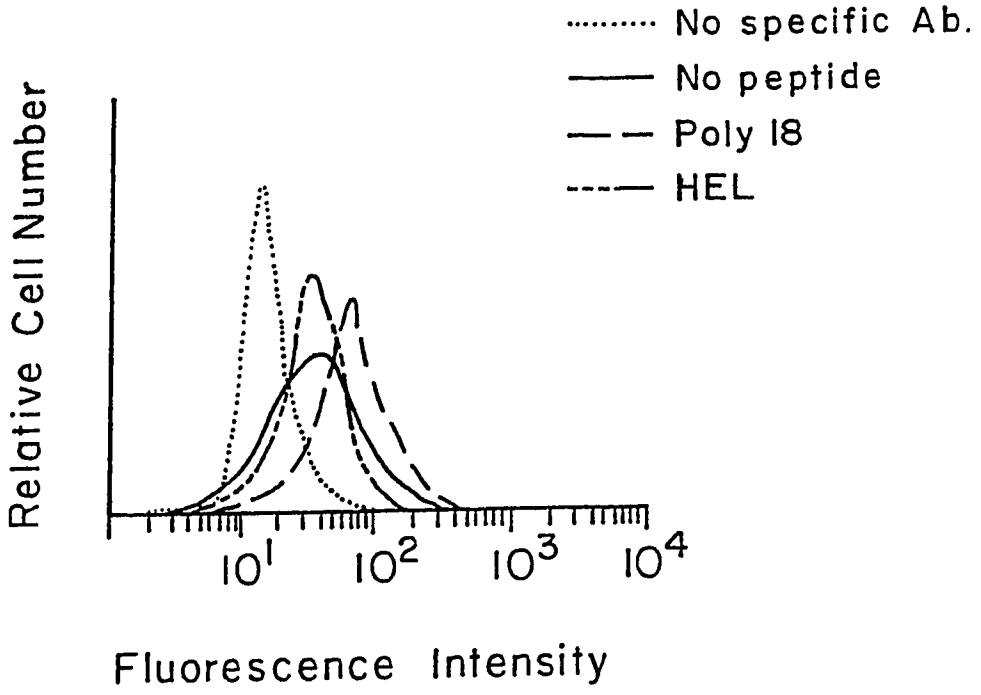

Figure 4.5 ***Exogenous I-A^d restricted peptide antigen Poly 18 increases cell surface expression of I-A^d.***
10^6 TA3 cells were incubated with (250 μg/ml) HEL or Poly 18 and without any added antigen for 24 hours. Levels of I-A^d on the cell surface of these cells was examined by FACS analysis (as described in Materials and Methods). The channels at which mean fluorescence intensity occured were 15 for background (sample without first antibody), 35 for control sample without our added antigen, 70 and 35 for the samples incubated with Poly 18 and HEL respectively.

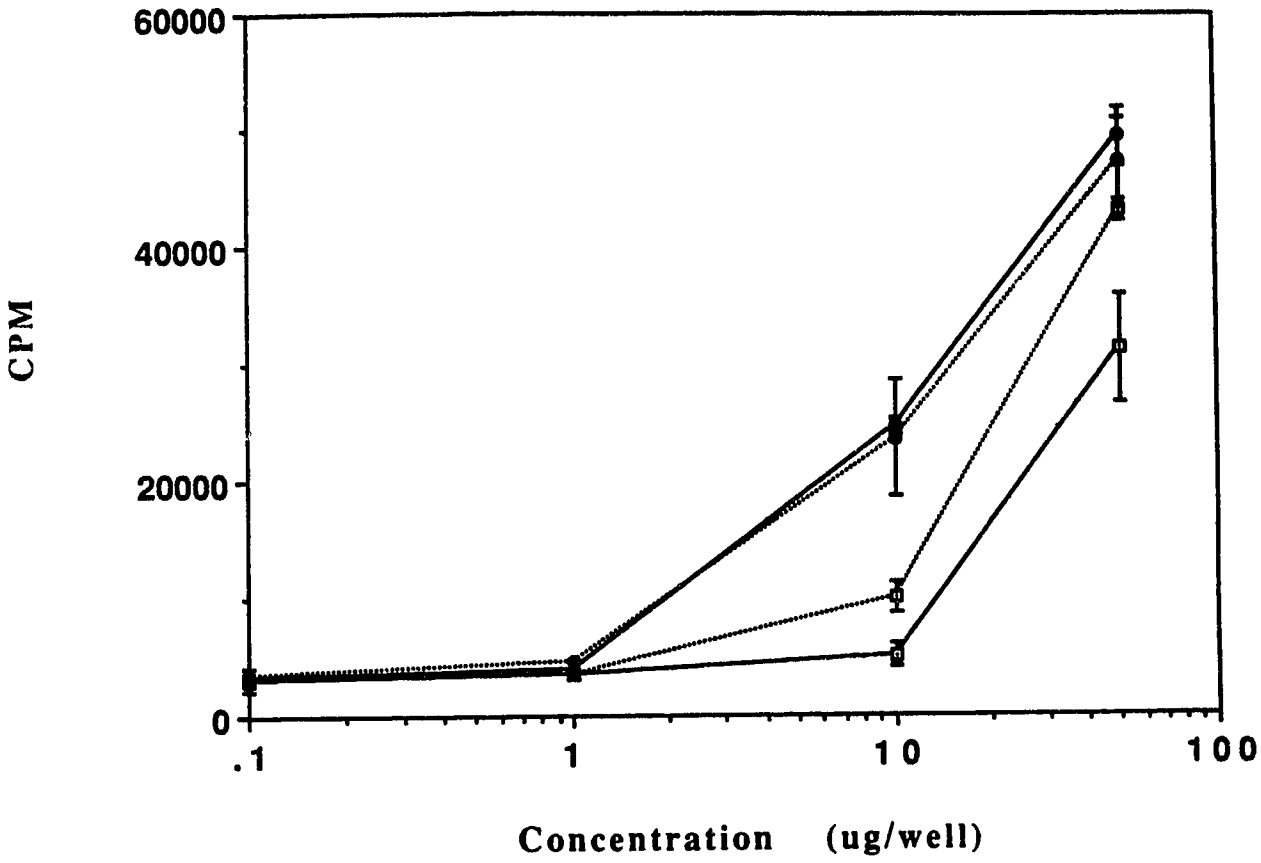

Figure 4.6 ***Antigen presentation of EYK(EYA)4 to A.1.1 T cell hybridoma.***

TA3 cells expressing high (●) and low (◻) levels of I-A^d molecules exhibiting mean fluorescence intensity at 100 and 40 channels respectively were used as APC. Fixed (—) or unfixed (·····) TA3 cells (5 x 10^4) were incubated with 10^5 T cell hybridoma cells with or without antigen EYK(EYA)$_4$ (1-50 μg/well). After 24 hours of culture, supernatant was collected and assayed for IL-2 content in a secondary culture (as described in Materials and Methods). The ^{3}H-Tdr incorporation (CPM) by the CTL-L in the absence of antigen was 2,852 ± 407.

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CHAPTER V

MECHANISM OF UPREGULATION OF CELL SURFACE EXPRESSION OF MHC CLASS II MOLECULES BY PEPTIDES

INTRODUCTION

MHC class I and class II molecules are cell surface glycoproteins involved in presenting antigenic peptides to T lymphocytes. The biosynthesis of class I and class II molecules initially follows that of other typical type I membrane glycoproteins. The biosynthetic pathway of class II molecules diverges from the constitutive secretory pathway and intersects the endocytic pathway (1). Immunocytochemical studies suggest that the class II molecules encounter the endocytosed material in late endosomes (2). The site at which the class II molecules associate with peptides has not been clearly established.

Experiments with mutant cell lines defective in class I expression, have demonstrated that the peptide has critical role in stabilizing the association of class I heavy chain-β2m complexes and in facilitating their movement to the surface (3-6). The mutant cell line appeared to produce unstable class I molecules which could be stabilized by culturing the cells at low temperature or with peptides (5).

In contrast to the role of antigenic peptides in stable association of MHC class I molecules, MHC class II αβ heterodimer can

assemble in the absence of antigenic peptide (7). However, experiments using purified class II molecules have suggested that stable binding of peptides is necessary for class II αβ dimers to enter into a compact state that resist dissociation in 1-2% SDS at room temperature (8). Subsequently, it has been found that conversion of class II dimers to the compact stable state is sensitive to chloroquine and occurs in molecules free of intact invariant chain (Ii). Only a fraction of newly synthesized class II molecules seem to be involved in peptide dependent conversion to a compact state (9).

In our previous experiments, we detected a TA3 cell (B cell hybridoma) variant, which expressed lower levels of class II I-A^d molecules on the cell surface. Addition of I-A^d-restricted peptides, e.g. I-Aβ^d 1-14, upregulated the expression of I-A^d on the surface of TA3 cells (Chapter IV). We have now attempted to investigate the mechanism of upregulation of I-A^d expression by I-Aβ^d 1-14 peptide. The induction of I-A^d expression by peptides seems to involve active metabolic processes and requires internalization of immunogenic peptide into the cells. Inhibition of protein synthesis does not affect the upregulation of I-A^d expression, whereas neutralization of intracellular acidic compartments seems to inhibit the induction of I-A^d expression by I-Aβ^d 1-14 peptide. These results suggest that the peptide fragments of MHC class II molecules may have a partial role in regulating the stability and expression of MHC class II molecules in the absence of exogenous antigenic peptides. This observation can be extended to the role of other self peptides in influencing the normal or abnormal expression

of MHC molecules in disease conditions such as MHC class II-linked autoimmune disorders.

MATERIALS AND METHODS

Peptides: Peptides were prepared by the Merrifield Solid-phase technique on a Beckman 990C Peptide Synthesizer (Palo Alto, CA) according to previously reported procedures (10).

Hybridomas: TA3 cells were obtained from Dr. L. Glimcher, Harvard Medical School, Boston. These are surface IgM^+, $I\text{-}A^{d,k}$ and $I\text{-}E^{d,k}$ expressing B cell hybridoma derived from the fusion of lipopolysaccharide stimulated (BALB/c X A/J)F1 spleen cells with the BALB/c ($H\text{-}2^d$) B cell lymphoma (11). The low $I\text{-}A^d$ expressing TA3 cell variants were obtained by culturing the cells *in vitro* for more than six months as described (Chapter IV). High and low $I\text{-}A^d$ expressing TA3 variants were selected by cloning by limiting dilution and screening subclones for $I\text{-}A^d$ expression on the cell surface by FACS analysis.

Culture Medium: RPMI 1640 medium (GIBCO laboratories, Grand Island, NY) containing $5X10^{-5}$ 2-ME, 10 mM HEPES, 2 mM Glutamine, 1.0 U/ml penicillin-streptomycin (GIBCO) was used with 10% heat inactivated Fetal Bovine Serum (FBS) (BDH Inc., Edmonton, Canada).

Monoclonal Antibody: Hybridoma cells producing the anti $I\text{-}A^d$ (MKD6) and anti B220 (RA3.3A1) were purchased from ATCC, Rockville, Maryland. MKD6 MAb used in these experiments was used

as ascites and anti B220 MAb was used as ammonium sulfate cut culture supernatant.

Chemicals: Cycloheximide and chloroquine were obtained from Sigma Chemical Company (St. Louis, MD). Brefeldin A (BFA) was obtained from Epicenter Technologies (Madison, WI). The TA3 cells (10^6/ml) were preincubated with or without cycloheximide (Chx, 20 μM), BFA (3 μM) or chloroquine (Clq, 150 μM) for 4 hours (12) before addition of the I-Aβ^d 1-14 peptide. The cells were cultured in continuous presence of inhibitors with the peptide for 18 h.

Immunofluorescence Analysis: TA3 cells (10^6) were stained with anti I-A^d or anti B220 MAb and FITC conjugated second antibody (Fab fragments only, TAGO Inc. Burlingame, CA, USA) according to previously reported procedures (Chapter IV). Prior to staining for FACS analysis, 10^6 TA3 cells were incubated in different conditions, in the presence or absence of I-Aβ^d peptide.

Fluorescence profiles generated by MAb binding to TA3 cells was analyzed on a FACScan (Becton Dickinson) in which a laser was used to excite FITC. Files were collected for 5000 cells from each sample, and measure of side scatter and forward scatter were stored along with fluorescence profiles. Data was plotted as the number of cells (ordinate) vs the log fluorescence intensity. The fluorescence profiles generated by antibody binding to TA3 cells were symmetrical curves. The fluorescence intensity of staining with second antibody is fluorescein (FITC)-conjugated goat anti-mouse Ig or FITC-conjugated goat anti-rat Ig was always lower than that

observed when TA3 cells were treated with both first and second antibody. Each experiment was repeated 2-3 times unless otherwise mentioned.

RESULTS

1. *Cloning of low I-A^d expressing TA3 cells by limiting dilution.*

TA3 cells when cultured *in vitro* for approx. twenty weeks showed low expression of I-A^d on their surface (Chapter IV). Upon cloning of these low I-A^d expressing TA3 cells, we were able to detect TA3 variants with high, low or intermediate I-A^d expression (Fig. 5.1) as determined by fluorescence profiles generated by binding of the anti I-A^d MAb. The expression of B220, I-A^k, I-E^{d,k} did not vary among the TA3 subclones with varying expression of I-A^d (data not shown) as detected by binding of the anti I-A^k, anti I-E^{d,k} or anti B220 MAbs.

2. *Effect of temperature on the surface expression of I-A^d on low I-A^d expressing TA3 clones.*

In order to examine if culturing the TA3 cells at reduced temperatures induces the expression of I-A^d on the cell surface, in a manner analogous to class I molecules (5), we exposed the low I-A^d expressing TA3 cells to varying temperatures for 24 hours and examined the cell surface expression of I-A^d (Fig. 5.2). As shown in the figure 5.2, there was some increase in the cell surface expression of I-A^d upon culturing the cells at low temperatures. There was an

apparent increase in the cell surface Ig expression as detected by binding of the FITC conjugated second antibody (Fig. 5.2). However, the net increase in the cell surface expression of I-A^d seems to be small and insignificant.

3. ***I-Aβ^d 1-14 peptide induces the expression of I-A^d on the cell surface at 37^{0}C but not at low temperature.***

We have previously observed that I-Aβ^d 1-14 peptide which has high affinity for intact I-A^d, induces an increase in the expression of I-A^d on the cell surface (Chapter IV). To determine if the induction of I-A^d by the I-Aβ^d 1-14 peptide is temperature dependent, we incubated the low I-A^d expressing TA3 cells in the presence or absence of I-Aβ^d 1-14 peptide at 9^{0}C and at 37^{0}C. The results shown in fig. 5.3 demonstrates that I-Aβ^d 1-14 peptides induced the expression of I-A^d on the cell surface to ~150% of the initial at 37^{0}C. There was no effect of the peptide on I-A^d expression at 9^{0}C. A B cell surface molecule B220, unrelated to I-A^d, was not affected by the presence of I-Aβ^d 1-14 peptide even at 37^{0}C (Fig. 5.3).

4. ***The effect of peptide on I-A^d expression on the cell surface is reversible.***

The culture of low I-A^d expressing TA3 cells in the presence of I-Aβ^d 1-14 peptide (125μM) led to a marked increase in the expression of I-A^d on the cell surface. The effect was not seen within the first 2-3 h of culture (Fig. 5.4a). There was a significant increase in I-A^d expression in 3-5 h of incubation in the presence of

the peptide. However, culturing the cells in presence of the peptide for longer period of time ie. 24 h did not lead to further increase in I-A^d expression. We further examined whether the effect of I-Aβ^d 1-14 peptide on I-A^d expression leads to peptide dependent changes or to other effects which do not need the continuous presence of the peptide. We cultured the low I-A^d expressing TA3 cells in presence of 125 μM of I-Aβ^d 1-14 peptide for 24 h. The peptide was then removed and cells were cultured for varying periods of time. The I-A^d expression on the cell surface (Fig. 5.4b) reduced with time. In 4 hours of culture, almost all the peptide-induced I-A^d molecules disappeared from the cell surface. The expression of B220 molecules on the cell surface remained unchanged under similar experimental conditions (Fig.5.4c).

5. ***Effect of cycloheximide (Chx), chloroquine (Clq), and brefeldin A (BFA) on the induction of I-A^d expression by peptides.***

To determine whether the induction of I-A^d cell surface expression by the I-Aβ^d 1-14 peptide requires new protein synthesis, transport from ER or acidic endosomal compartments, we examined the effects of various inhibitors on the induction of I-A^d by peptide. The results (Fig. 5.5a) showed that Chx (protein synthesis inhibitor) and BFA (inhibitor of intracellular transport from ER to Golgi) partially imhibited the induction of I-A^d surface expression by I-Aβ^d 1-14 peptide. A lysosomotropic agent, chloroquine inhibited the I-Aβ^d 1-14 peptide induced expression of I-A^d (Fig. 5.5a). We used the B220 expression on the cell surface as

controls where the TA3 cells were treated similarly in the presence or absence of various inhibitors and examined for B220 expression on the cell surface (Fig.5.5b).

DISCUSSION

In vitro culturing of TA3 cells for twenty weeks of time resulted in a specific loss of cell surface of I-A^d. One of the reasons for this reduction could be attributed to the loss of specific self peptide ligands, resulting in the expression of unstable MHC class II heterodimers (Chapter IV), (9, 8). The loss of I-A^d expression could be partially corrected by the addition of exogenous peptide ligands having high affinity for intact I-A^d. The cloning of the low I-A^d expressing TA3 cells yielded three categories of subclones expressing high, intermediate and low levels of I-A^d on the cell surface. The low I-A^d expressing TA3 subclones have been used in this study. We have found great variability and instability in I-A^d expression on various subclones of TA3 cells. The high I-A^d expressing TA3 subclones become low I-A^d expressing upon further culturing *in vitro*, the reverse has been found to happen with some of the low I-A^d expressing subclones. Although, not clearly explained, it seems cell cycle stages, cell concentrations and culture conditions may lead to the observed behavior. Apart from the aforementioned variability, our studies demonstrated a few consistant findings, giving an insight into the role of immunogenic peptides in regulating the cell surface expression of MHC class II molecules and the mechanism involving this regulation.

It has been previously demonstrated that culturing the cells at low temperature induces association, stability and expression of empty MHC class I molecules (5). Such empty MHC class I molecules can be stabilized by exogenous peptides in an allele-specific manner. Our experiments with MHC class II, I-A^d expression suggest that there is an increase in I-A^d expression in the cells which are cultured at low temperature. In contrast to class I expression (5), where 4-5 fold increase was observed at 26^0C, we could find less than a 2-fold increase in the class II expression on the surface of cells cultured at 22^0C or 9^0C for 24 hours. The observation is further underscored by the findings that B cell surface Ig molecules also increase on the cell surface, when the cells were cultured at lower than physiological temperatures. Lowering the temperature of cells leads to inhibition of the degradation of microinjected as well as endogenous proteins (13). We could not examine whether the increase in surface expression of I-A^d was due to change in the conformation of the antibody binding epitope, due to lack of an epitope specific MAb. The idea of stabilization of empty class II molecules at low temperature can not be put forward as it has been established that empty MHC class II molecules can be expressed on the cell surface at physiological temperature (7) and is not supported by our data (fig. 5.3).

The experiment involving incubation of low I-A^d expressing TA3 cells in the presence of I-$A\beta^d$ 1-14 peptide, at different temperatures ie. physiological (37^0C) and low (9^0C), however suggested the peptide induced I-A^d expression requires physiological temperatures. This can also be interpreted to suggest

that the peptide ligands have to be internalized into the cells in order to show an inductive effect on the class II expression. At low temperature (ie. 9^0C), endocytosis is reduced (14) and the membrane fluidity is also decreased (15), thus decreasing the efficiency of peptide uptake into the cells. The requirement of internalization of the peptide ligand to induce the I-A^d expression on the cell surface has also been suggested from our previous experiment (Chapter IV) using glutaraldehyde fixed TA3 cells (Fig. 4.6).

TA3 cells have been demonstrated to exhibit rapid turnover of peptide-MHC class II complexes. At the same time Ia molecules in these cells have been shown to possess long half-lives (17). These two characteristics will lead to rapid exchange of peptides and at the same time rapid degradation of the peptide ligands which are already bound to the MHC molecules. In the kinetics experiment we saw a rapid increase in I-A^d expression, induced by the I-Aβ^d 1-14 peptide, during 3-5 hours of incubation. The removal of exogenous peptide from the medium led to a linear decrease in the induced I-A expression. These results suggest that the half-life of the peptide I-Aβ^d 1-14/I-A^d complex is in the range of =2hours. This measurement is limited and can not be extrapolated directly to kinetic analysis because the reduction in the I-A^d expression after removal of peptide ligand has not been directly demonstrated to be due to the decay of I-A^d/peptide complexes. Furthermore, this analysis can not be generalized to address the association/dissociation of other peptide ligands and I-A^d; as the turnover rates of different peptides bound to I-A^d molecules could

vary. The half life of HEL (46-61) peptide/I-A^k complexes in TA3 cells has been estimated to be 15-53 min (16).

The intracellular location where the peptide I-$A\beta^d$ 1-14 may affect the I-A^d expression seems to be the endosomal compartments because the effect of peptide on I-A^d expression could be inhibited by chloroquine (a lysosomotropic agent). There was a partial inhibition in the induction of I-Ad expression by the peptide in the presence of CHX and BFA. It can be suggested that pretreatment of cells with CHX or BFA for 4 hrs may have reduced the available intracellular pool in the endosomal compartments. The experiments using Chx and BFA also suggest that I-$A\beta^d$ 1-14 peptide may interact with the recycling I-A^d $\alpha\beta$ heterodimer and result in the formation of a compact form followed by an enhanced efficiency of I-A^d expression on the cell surface. This interpretation does not imply that all of the recycling MHC class II heterodimer (ie. I-A^d molecules) can interact with the added peptide ligand I-$A\beta^d$ 1-14. It seems that only a subpopulation of recycling I-A^d molecules can interact with the exogenous peptide ligand and the remaining molecules can either interact with other endogenous ligands or can be expressed on the cell surface in the absence of a ligand.

In conclusion, our study demonstrates that distinct peptides can influence the expression of MHC molecules. The nature of specific peptides expressed by class II-bearing cells may have a significant influence on T cell development, self tolerance and alloreactivity.

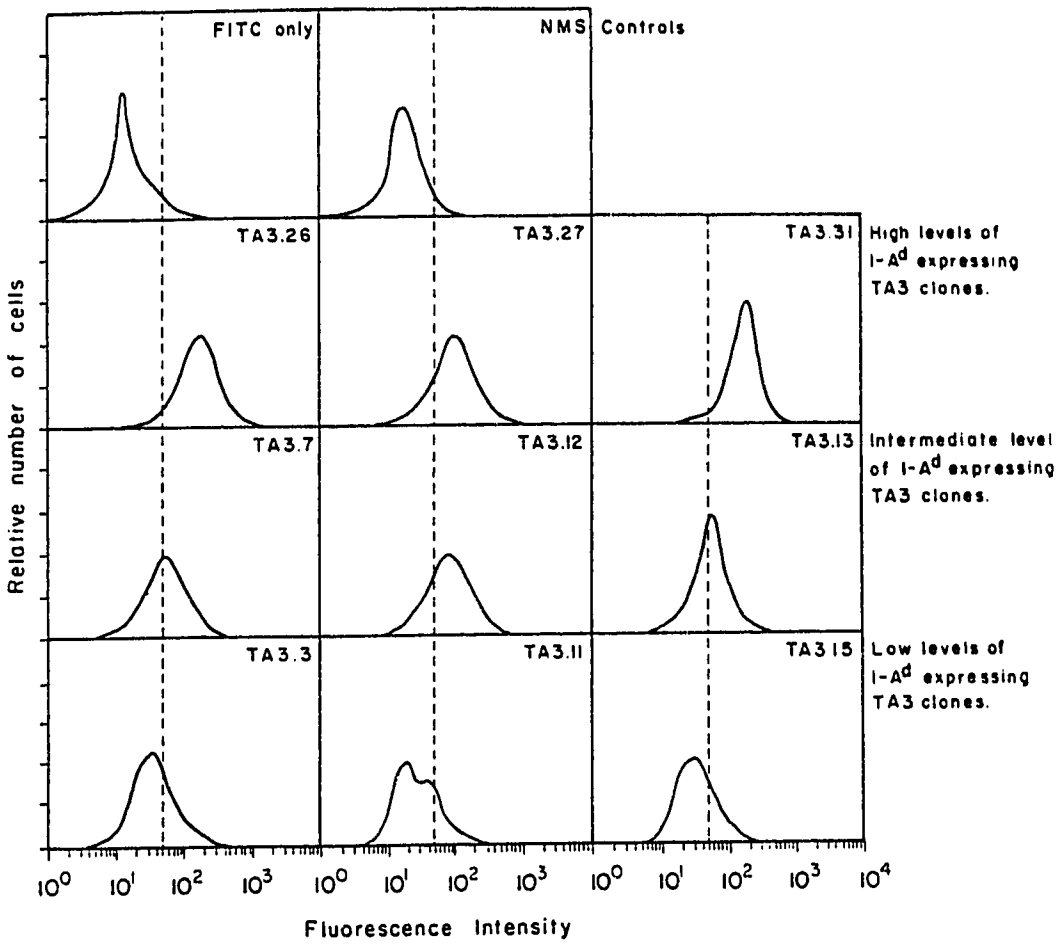

Fig. 5.1 ***Immunofluorescence analysis of I-A^d surface expression on TA3 variant clones.***

The fluorescence profiles generated by binding of the second antibody ie. FITC conjugated goat anti mouse Ig was almost overlapping for all of the clones, as represented in the first panel.

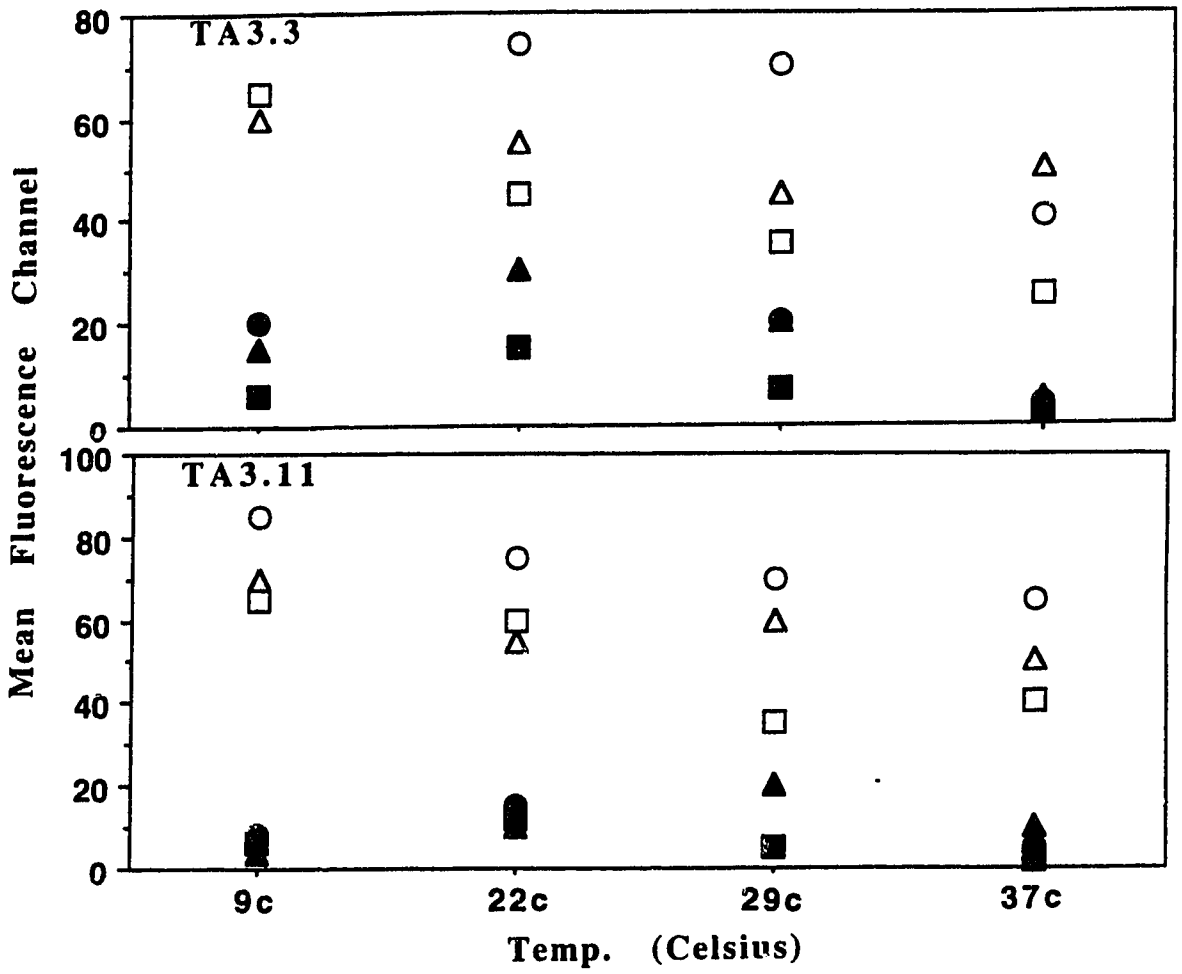

Fig. 5.2 ***Effect of low temperature on I-A^d expression.*** Data with two low I-A^d expressing TA3 clones (upper and lower panel) is shown. Three experiments are denoted by (▲), (○) and (■) in each case for both I-A^d expression (open symbols) and the surface Ig expression (ie. second antibody binding) (dark symbols).

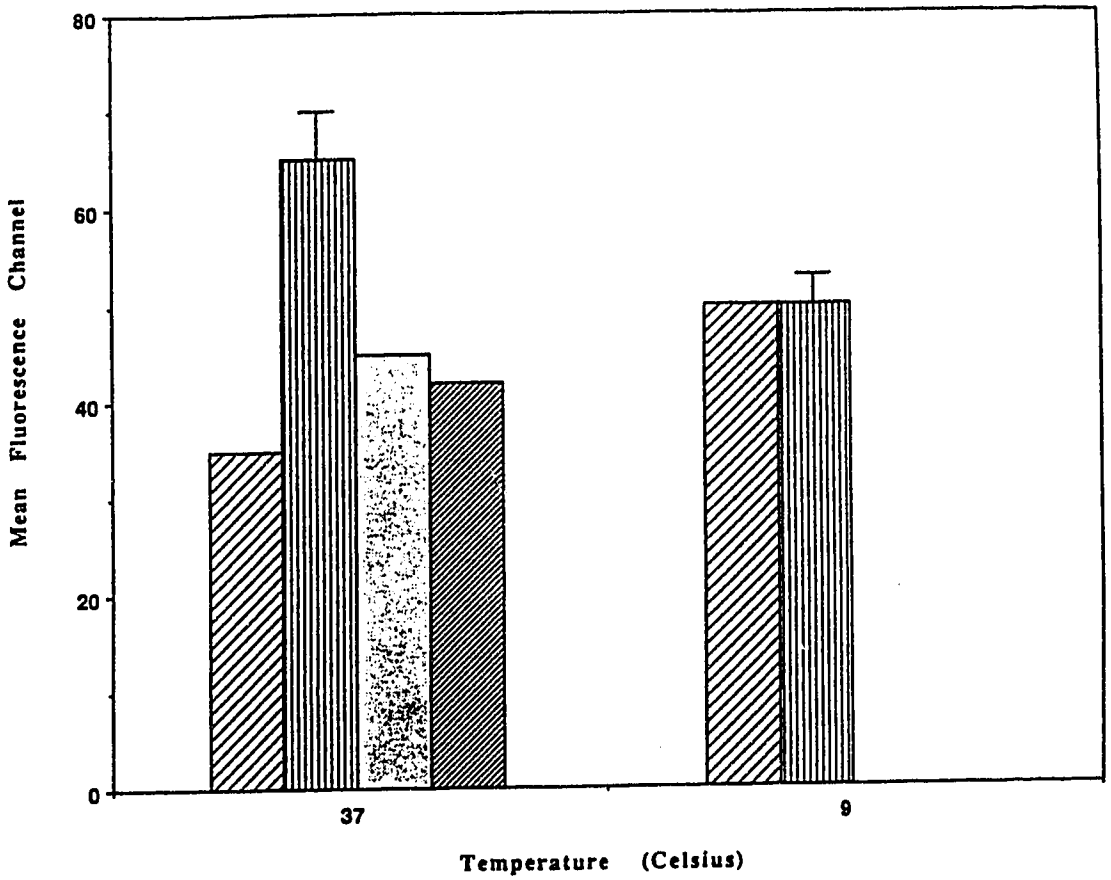

Fig. 5.3 ***I-Aβ^d 1-14 peptide induces the upregulation in I-A^d surface expression at 37^{0}C but not at 9^{0}C.***

TA3 (10^6) cells were cultured in presence (▥) or absence (▨) of 125 μM I-Aβ^d 1-14 peptide for 24 h and stained for I-A^d expression. B220 expression of TA3 cells cultured in presence (▩) or absence (▨) of I-Aβ^d 1-14 peptide (125 μM) at 37^{0}C is also shown. This figure represents one out of two repeated experiments

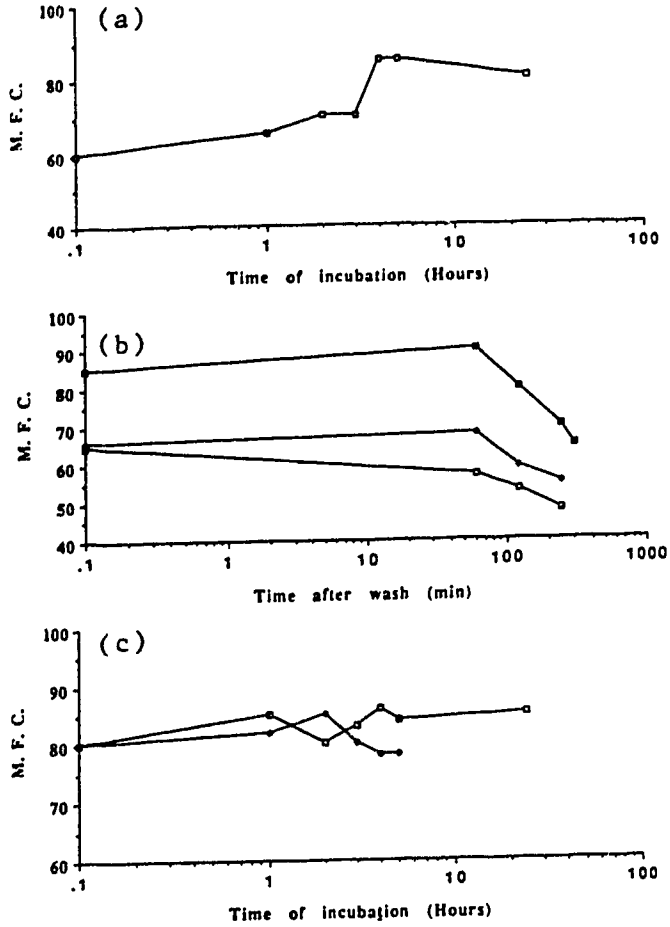

Fig. 5.4 ***The effect of I-Aβ^d 1-14 peptide on I-A^d expression can be reversed.***

(a.) TA3.3 cells (10^6) were incubated in presence of I-Aβ^d 1-14 peptide (125 M) for varying periods of time and examined for I-A^d expression. (b.) TA3.3 cells (10^6) were incubated for 24 h in presence of I-Aβ^d 1-14 peptide (125μM). The cells were washed thrice and cultured for varying periods of time. Three lines represent three different experiments. (c.) TA3.3 (10^6) cells were incubated for varying periods of time in the presence of I-Aβ^d 1-14 peptide (125 μM) and stained for B220 expression (—⊡—). Line (—◆—) represents the TA3 cells cultured for various periods of time after removing the I-Aβ^d 1-14 peptide. M. F. C. represents the mean fluorescence channel of the fluorescence intensity.

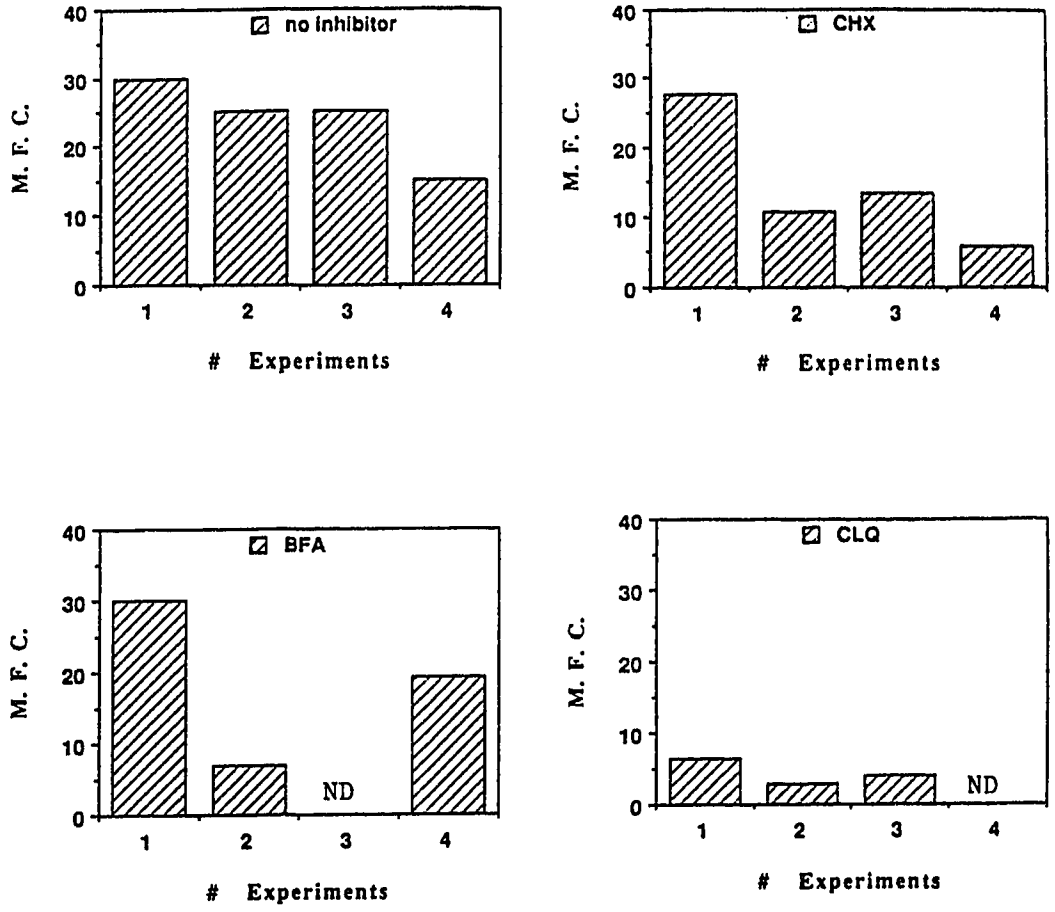

Fig. 5.5a ***The Effect of Chx, Clq, and BFA on the peptide induced expression of I-A^d.***

TA3 cells, preincubated with or without the inhibitor were cultured in presence or absence of I-Aβ^d 1-14 peptide for 18 h and stained for I-A^d expression on the cell surface. The mean fluorescence channel of the sample without the peptide has been subtracted from the M.F.C. of the sample incubated in the presence of the peptide (125 μM) before plotting on the graph.

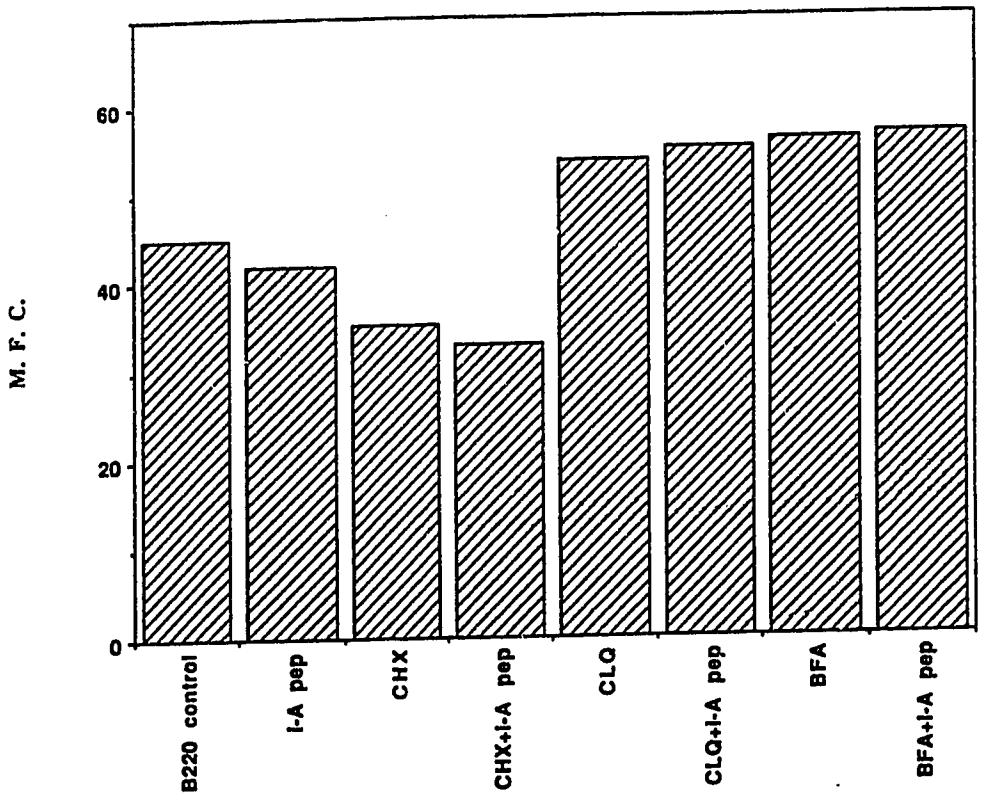

Fig. 5.5b. ***The Effect of Chx, Clq and BFA on B220 expression.***

TA3 cells were treated in a manner similar to fig. 5.5a in the presence or absence of I-Aβ^d 1-14 (125μM) peptide and examined for B220 expression.

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CHAPTER VI

GENERAL CONCLUSIONS

In this thesis I have investigated the immunological recognition of self MHC class II molecules and the regulation of their expression on the cell surface. My first objective was to examine the processing, presentation and recognition of self MHC class II molecules. My second objective was to extrapolate these results and determine the role of processed MHC class II molecules in regulating an immune response. I have used synthetic peptides corresponding to the most polymorphic regions of MHC class II α and β chains as representative of processed fragments of MHC class II molecules. The results are summarized and discussed below.

The presentation and recognition of self MHC class II peptides

We examined the affinity of synthetic I-A^d peptides towards the ag binding groove of syngeneic I-A^d molecules in order to investigate if it is possible that the I-A^d peptides can be presented by self APCs in the context of self I-A^d molecules. The competitive inhibition of stimulation of ag reactive T cells by I-A peptides was used as the approach. The rationale was that if the self I-A peptides have an affinity for syngeneic I-A, they would compete with the ag in the activation of T cell hybridomas when

added to the culture. Most of the I-Aβ^d and I-Aα^d peptides inhibited the activation of I-A^d restricted Poly 18 specific T cell hybridoma A.1.1. The inhibition by various peptides varied from 50-90% of the control and suggested the differences in the affinities of various I-A peptides for intact I-A^d molecules on the APC. I-Aβ^d peptides seemed to have higher affinity than I-Aα^d peptides. The inhibition of T cell stimulation in these experiments by I-A peptides could be either due to the competition between the ag and I-A peptide for the ag binding groove of I-A^d or due to the competition between the I-A peptide and the intact (I-A^d) MHC class II molecules to interact with the TCR of the T cells. We found that the inhibition of activation of T cells by I-A^d peptides was due to the competition between the ag and I-A^d peptides for the ag binding site on the MHC (I-A^d) molecules. In the experiment where APCs were prepulsed with ag and fixed and added to the T cells and I-A^d peptides, no inhibition in the T cell stimulation was observed. In contrast, in the experiment where APCs were prepulsed with I-A^d peptides before fixing with glutaraldehyde then added to T cells and antigenic peptides, considerable inhibition in the T cell stimulation was observed. Other reports have also suggested that fragments of MHC class II molecules can be presented by syngeneic MHC class II molecules (1,2).

These results prompted us to examine the immune recognition of the I-A^d peptides in syngeneic mice. We examined the proliferative response of naive syngeneic T cells towards the

synthetic peptides of self I-A^d molecules from both $\alpha 1$ and $\beta 1$ domains. A number of these peptides were found to be stimulatory. The stimulation of T cell proliferation correlated with the ability of the peptide to bind to the ag binding groove of I-A^d in most cases except with the I-$A\beta^d$ 62-78 peptide. These results suggested that the syngeneic T cells can be primed towards self I-A^d peptides *in vivo*. Upon immunization with I-A^d peptides, the response of primed T cells was found to be increased towards some peptides and with others the response was similar to that of unprimed T cells. The response of T cells towards most of the I-A^d peptides could be inhibited by anti I-A^d (MKD6) MAb, further suggesting the presentation of these self I-A^d peptides in the context of I-A^d molecules on the APC. The self peptide I-$A\beta^d$ 62-78 was found to be nonstimulatory for both primed and unprimed syngeneic T cells. The reason could be either the self tolerance against this epitope of I-A molecule or the induction of regulatory cells by this peptide. As a control we examined the T cell response of mice allogeneic towards I-A^d peptides. These mice responded well to almost all of the I-A^d peptides upon priming. These studies suggested that the syngeneic T cells may become primed to fragments of self I-A^d molecules probably due to endogenous processing and presentation of self I-A^d molecules. This conclusion is in agreement with the hypothesis put forward by Kourilsky et al (3) that most self cellular proteins are processed and presented as peptides in the context of class I or class II MHC molecules for interaction with T lymphocytes. Likewise, in the adult animal, it has been shown previously that

self molecules such as hemoglobin (4) or MHC class II molecules in humans (2, 5) are regularly processed and presented to induce T cell responses in the periphery. The self peptides, which are continuously presented at the cell surface of the APC, may contribute to the acquisition of the T cell repertoire in ontogeny and to the regulation of the T cell response (6-8). Both of these aspects were the targets of our further investigations.

We examined the development and progression of T cell reactivity and tolerance to self I-A peptides in mice. We found that naive T cells from neonatal BALB/c mice did not respond to any of the self I-A^d peptides. However, by day 11, the response of naive T cells was almost similar to that observed in adult mice but there was no reactivity to the self peptide I-$A\beta^d$ 62-78. In contrast, T cells from old BALB/c mice proliferated in response to self I-$A\beta^d$ 62-78 peptide. A few other strains of mice showed a similar reactivity pattern to self I-Aβ 62-78 peptide. The adult mice responded to the allo I-Aβ 62-78 peptide after priming and thus showed specific non reactivity to self I-Aβ 62-78 peptides. This region corresponding to the peptide I-Aβ 62-78 has been implicated in contacting the TCR in the recognition of MHC-peptide complex (9) and has been hypothesized to be on the α-helix forming the wall of the ag binding groove and exposed to the TCR (10). It is possible that the region represented by I-Aβ 62-78 peptide is exposed to developing thymocytes and T cells reactive this region are deleted. On the other hand, immune reactivity to other self I-A^d peptides presents a dilemma. First, it

suggests that the T cells reactive to self I-A^d peptides escape from negative selection process during thymic maturation probably because these self I-A^d peptides are not presented in this form in the thymus and T cells reactive to self I-A peptides are not deleted. Alternatively, T cells having intermediate affinity for self I-A peptides/MHC class II complex get positively selected in the thymus. In other words, MHC restriction of T cells would require that the T cells have some affinity for self MHC molecules. Reactivity of T cells towards self MHC class II peptides and intact class II molecules *in vivo* may be tightly regulated so as to avoid any outcome as autoimmunity. Apart from cell-cell contact, it appears that most T cell responses are manifestations of the biological activities of cytokines produced by these cells. Mature $CD4^+$ T cells are intrinsically heterogeneous in function. In addition, the external environment can influence the relative amounts of lymphokines that a T cell secrets upon stimulation. It has also been suggested that early in ontogeny, there are factors that are favorable to the induction of a protective TH1-type lymphokine repertoire and that this bias is maintained in the adult because the auto aggressive response does not break through (11). In *in vitro* culture, the intricate balance of the cytokines produced may be disrupted resulting in an apparent self reactivity. It is important to recognize that the apparent T cell reactivity to self I-A peptides and self I-A molecules is probably conferred by the T cells having intermediate affinity for self MHC class II molecules, as the T cells with high affinity would be expected to be negatively selected during development. In our

studies, we did not observe T cell reactivity towards self I-A^d peptides in T cells isolated from neonatal (1d-3d old) BALB/c mice. It is possible that the frequency of T cells reactive to self I-A^d peptides is low in 1d-3d old mice, thereby no apparent proliferative response can be observed. It is also possible that the presence of regulatory cells in the T cell population of neonatal BALB/c mice (1d-3d old) led to the observed behavior. We have not resolved these issues in our studies.

Immunoregulatory role of self I-A peptides

The interpretation that self I-A^d peptides may be generated *in vivo* prompted us to investigate their immunomodulatory role. It has been demonstrated earlier that self-derived peptides can compete efficiently *in vivo* to block a T cell response directed against a foreign element (12). Several groups have also modulated the response to autoantigens involved in autoimmune diseases by using peptides capable of competing for MHC recognition (13-16). The presentation and immunorecognition of an ag is dependent upon the level of MHC expression on the cell surface. We observed that the MHC class II I-$A\beta^d$ peptide, with high affinity for intact self MHC class II molecules, could influence the expression of I-A molecules. The ability of peptides to upregulate the expression of I-A^d correlated with the affinity of the peptide with intact I-A^d. It can be indirectly inferred from our data that peptides may influence an immune response by regulating the MHC expression on the cell

surface (fig. 4.6). In our experiments, cycloheximide and brefeldin A partially inhibited the induction of I-A^d expression probably because the treatment of cells with CHX or BFA reduces the intracellular available pool of $\alpha\beta$ heterodimer for association with peptide. The induction of I-A^d expression by peptide could be inhibited by chloroquin. Therefore, the mechanism of upregulation of I-A^d expression by peptides seems to involve an intracellular endosomal compartment.

It can be speculated that *in vivo* a vast number of self peptides may be available to interact with a single class of MHC molecule (17), the additive effect of the self peptides may lead to significant regulation of MHC expression on the cell surface. Newly synthesized MHC class II-Ii complexes leave the ER and move through the Golgi and trans-Golgi network where a signal in the Ii chain redirects the complex to the endosomal organelles. This diversion of MHC class II molecules from the classical export pathway leads to slow rate of transport and a better opportunity for coordinated processing of the antigen and of Ii. Proteolytic cleavage and subsequent dissociation of Ii occurs in endosomal compartments, allowing the peptides from endocytosed proteins to bind to the released class II molecules (18, 19). It has been suggested by Germain and colleagues that newly synthesized MHC class II molecules behave like partially folded proteins, with peptide acting as adopted portion of the MHC polypeptide structure that is necessary for the completion of conformational maturation (20). The self peptides may also competitively inhibit

each other or the foreign peptide antigens in influencing the expression of the MHC molecules, or their presentation leading to the modulation of immune responses.

Future Directions

This study has answered a few specific questions regarding the immunological recognition and regulation of expression of self MHC class II molecules. The answers have inspired many more questions which can be explored in future analyses including:

1. We have explored the immunological recognition of self MHC class II molecules, viewed with respect to one arm of the immune system. In order to examine the complete immune reactivity to self MHC class II molecules, the B cell reactivity and humoral response to self I-A peptides can be explored. These two studies together will be important to the understanding of the self reactivity and regulation thereof.

2. The frequency of T cells reactive to the self I-A peptides is an important question. The comparison of frequency of self I-A reactive T cells in normal and MHC linked autoimmune conditions may give an insight in to the role of self I-A reactive T cells in the development of autoimmune disease condition. This can also be approached in a different manner. T cell lines and clones reactive to self I-A peptides can be generated and their role in protecting or evoking an autoimmune disorder can be

examined. For this purpose, T cell lines reactive to the region of MHC molecules, which are found to be directly linked to an autoimmune disorder will be of potential. In autoimmune NOD mice, T cell lines reactive to I-Aβ^{NOD} (encompassing the region 56-60 aa residues) may be generated and examined for their immunomodulatory role. A similar approach can be used to modulate other MHC class II-associated autoimmune diseases.

3. In our study, we have suspected the presence of regulatory cells in neonatal mice as well as adult mice. The T cells can be fractionated on the basis of their phenotype ($CD4^+$ or 8^+) or function (on the basis of their secreted cytokine pattern). These fractionated T cells can be subdivided on the basis of stimulation or inhibition of the T cell responses to self I-A peptides. These stimulatory or inhibitory regulatory cell populations can also be used to modulate ag specific immune response *in vivo*. The regulatory cells may be the outcome of previous immunological experiences of the animal. In order to examine this possibility, specific pathogen free and specific infected mice can be used in the above mentioned experiments and compared for the presence or absence of the regulatory cells and their influence on the T cell reactivity to self I-A peptides.

4. The *in vitro* inhibition of T cell stimulation by I-A peptides can also be interpreted to have a potential in regulating the T cell response to foreign or self peptide antigens *in vivo*. We can explore whether coimmunization of a peptide ag with self I-A

peptides will have an influence on the T cell reactivity to the peptide ag.

5. The affinity of self I-A peptides towards the ag binding groove of syngeneic MHC class II molecules can be determined by direct binding assay. This kind of analysis may give an actual estimate of the number of I-A peptides bound to a single cell and may quantitatively measure the regulation of I-A expression. It can further be determined whether the binding of self I-A peptides can be competed out by the presence of other peptide antigens. These experiments will provide a potential for modulating the immune response by I-A peptides in a quantitative manner.

6. The role of self I-A peptides in syngeneic MLR can be examined more directly. T cell lines reactive to the syngeneic APC can be generated and examined for cross reactivity to self I-A peptides in the context of MHC class II molecules. Similarly, these studies can be extrapolated to examine the role of I-A peptides in allo MLR.

7. The role of self I-A peptides in positive and negative selection of T cells can also be determined. Mice transgenic for the selective expression of the self I-A peptide in the thymic epithelial cells can be generated. For this purpose, DNA constructs can be designed so that they contain specific promoter for selective expression in the thymic epithelial cells and contain an open reading frame for the desired peptide with signal sequences to direct their expression in specific intracellular organelles. The frequency of T cells reactive to the self I-A

peptides can be determined and compared to that from the non transgenic mice.

In conclusion, strong T cell proliferative responses can be generated in normal mice against peptides derived from self MHC class II molecules. These peptides may be generated in vivo and may prime the T cells and thereby influence the T cell repertoire of an individual. In addition, these self peptides and the self peptides reactive T cells may have potential for immunoregulation.

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