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A novel pathway of presentation by class II-MHC molecules involving peptides or denatured proteins important in autoimmunity

James F. Mohan and **Emil R. Unanue**
Department of Pathology and Immunology, Washington University School of Medicine, St Louis, Missouri USA

Abstract

We describe here a pathway of presentation involving peptides or denatured proteins that generate unique peptide-MHC complexes. Such complexes select for non-conventional CD4 T cells. We have examined this pathway and the corresponding CD4 T cells in diabetic autoimmunity. Autoimmunity requires both the escape of self-reactive T cells from thymic selection and, importantly, suitable conditions in peripheral tissues that allow for activation of T cells. In the autoimmune diabetes of NOD mice, insulin reactive T cells are highly focused on a peptide, encompassing the 9–23 segment of the B chain (B:9–23) bound to I-A^{g7}. Examination of the B:9–23 reactive T cell repertoire revealed the presence of two independent sets of T cells that recognize this epitope. One set, called type A, reacted like conventional CD4 T cells, recognizing both processed insulin protein and soluble B:9–23 peptide presented by APC. These T cells were highly deleted in the thymus and poorly represented in the periphery. The second set, called type B, did not recognize processed insulin protein presented by APC, but reacted strongly to the presentation of soluble B:9–23 peptide. Notably, this set was not deleted in the thymus, abundant in the periphery and caused diabetes. Free insulin peptides generated a unique peptide-MHC complex not found after insulin processing. These two T cell subsets discriminated between two independent, overlapping registers found within the B:9–23 peptide. In the islets of Langerhans, beta cells constitutively generated proteolyzed peptides from insulin, which were taken up by intra-islet APC and presented to peptide-specific type B T cells. Thus, self-reactive T cells that escape thymic selection can become pathogenic in the target organ where high concentrations of antigen and/or differences in intracellular processing lead to the presentation of peptides bound in distinct registers from those found in the thymus.

Keywords

autoimmunity; antigen presentation; class II major histocompatibility molecules; T cells; type 1 diabetes

We have identified a novel pathway of presentation by Antigen Presenting Cells (APC) that involves free exogenous peptides or denatured proteins and that results in the generation of

Corresponding author: Emil R. Unanue, Department of Pathology & Immunology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110 USA, Phone: 1-314-362-7440, Fax: 1-314-362-1403, unanue@wustl.edu.

unique complexes of peptides with class II Major Histocompatibility Complex proteins (pMHC) (reviewed in Mohan and Unanue, 2012, which has extensive references to the issues discussed here). We made the paradoxical observations of CD4 T cells that recognized in APC a pMHC complex derived from a free peptide or a denatured protein. These T cells did not recognize the same segment of the protein when processed from the protein. We documented biochemically that the processing of the protein selected a similar stretch of residues. (We refer to the unique pMHC as a type B pMHC complex and the complementary T cells as type B; the conventional pMHC and T cells are referred as type A.)

These findings apparently contradict the basic rules of antigen processing established years ago in which T cells were shown to recognize equally well peptides from the protein processing or free peptides or denatured proteins. The type B T cells have been largely overlooked, yet as we now have shown in autoimmune diabetes, these cells can be a major component of autoimmunity to autologous proteins (Mohan et al., 2010, 2011).

The explanation resides in the handling of proteins and peptides by the APC. The handling of protein antigens by APC places restriction on the repertoire of peptides bound to MHC-II so that from a given segment of the protein, a limited distinct number of pMHC complexes can be generated. In contrast, free exogenous peptides offered to the APC do not suffer such constraints so that a given segment generates a more diverse series of pMHC complexes. The explanation in great part centers on the site of assembly of the pMHC: the protein traverses to a deep vesicle (the MIIC) where peptide editors like H2-DM eliminate the more unstable complexes. This editing does not take place with exogenous peptides in which binding to MHC-II takes place in sites where H2-DM is not present. The end result is that exogenous peptides generate a more varied pMHC repertoire. Thus, some of the T cells that recognized exogenous peptide will be reactive to the peptide but will not see the protein-derived epitopes (since these are edited off in MIIC).

The initial studies with HEL

We first identified this pathway when examining the repertoire of peptides from the model protein hen egg-white lysozyme (HEL). We had identified the major segment from HEL selected by the MHC-II molecules in APC using mass spectrometry approaches. In examining the repertoire of CD4 T cells to this dominant segment, we found both type A and B T cells, the latter being the ones that only recognized the peptides. A series of biochemical and cellular findings gave a cellular explanation for the findings: i) we isolated the peptide from the processing of HEL and then offered it to type B T cells as an exogenous peptide; in such a situation it was recognized, a key indication that the explanation resided in the manner in which the protein and peptides were being handled by the APC; ii) purified I-A^k molecules incubated with peptide stimulated both type A and B T cells, indicating that free peptides were able to create each pMHC complex; yet addition of H2-DM eliminated the type B pMHC, while the type A was preserved; other studies indicated that the type B complex were less stable and shorter lived; iii) cellular studies indicated that HEL was processed in a late vesicular compartment (the MIIC?) where both pMHC were generated, but in such a vesicle H2-DM edited off the more unstable type B pMHC; iv) in contrast, peptides were generated in recycling vesicles or plasma membrane where H2-DM was poorly represented, thus generating both A and B pMHCs. From these as well as other findings we concluded that type A and B were formed from the same peptide segment and were likely explained by conformational isomers of the peptide complex.

An added perspective on the meaning of the type B T cells was made examining mice made transgenic to HEL expressed under a class II promoter. These mice expressed HEL in APC

at a high level and added an important perspective. The APC from such mice mostly expressed type A pMHC. Transgenic mice immunized with HEL were completely unresponsive; as expected, clonal deletion was highly effective. However, a T cell response was generated when the HEL-transgenic mice were immunized with peptide. The T cells from these mice only recognized the peptide, but not HEL. We concluded that the type B had escaped thymic purging and peripheralized. This key experiment turned our attention to the possibility that such type B pMHC might be found in autoimmunity. We find this is the case in the autoimmune diabetes of the NOD mouse (Mohan et al., 2010).

Prior findings to our HEL studies

In retrospect, there are a number of observations made since the early studies on immunogenicity that are examples of the unconventional repertoire to denatured proteins or peptides. The early pioneering studies of Karl Landsteiner on immunogenicity of proteins, reviewed in his classical book *The Specificity of Serological Reactions* (the 1936 edition reproduced by Dover Publications), showed examples in which denatured autologous proteins, mostly albumins or immunoglobulins, induced antibodies, while the native proteins did not. More recently, in 1963, Benacerraf's group reproduced the findings, but with a different nuance: guinea pigs were found to develop delayed sensitivity, an exuberant T cell response in this species, to denatured gamma globulin, but never to the native protein (McCluskey et al., 1962). This is an observation akin to the studies on HEL transgenic mice described above.

Akin also to our findings were the studies by Eli Sercarz on crypticity. Sercarz classified cryptic epitopes as 'hidden' epitopes of the protein not expressed during processing of the protein. A series of reports went on to identify responses against cryptic peptides in animals tolerant to the whole protein. Later, many of these epitopes were shown to be presented on MHC class II molecules, and often in high concentrations after protein processing. Thus, an alternative explanation was needed for many of the T cells that recognized 'cryptic' epitopes.

Lastly, there are a number of reports in the literature, discussed in our recent review (Mohan and Unanue, 2012), of responses to a number of self-peptides in clinical autoimmune diseases. At face value, the responses were compatible to type B T cells, however the significance and role of them has not been evaluated.

The findings in NOD diabetes

Insulin, the major protein of the beta cell secretory granule, is a dominant autoantigen in NOD diabetes, a finding championed by the extensive studies emanating mostly from the laboratory of George Eisenbarth (reviewed in Mohan and Unanue, 2012). Many of the insulin-reactive CD4 T cells recognize an immunodominant segment of the B chain, residues 923 (B:9–23). The B:9–23 segment binds weakly to I-A^{g7} with a fast dissociation rate.

We found two sets of the insulin-specific CD4 T cells, each falling into the type A and type B criteria. The type A T cells recognized APCs pulsed with insulin protein or the B:9–23 peptide. However, most responded to APCs pulsed with B:9–23 peptide, but did not respond to insulin. In adoptive transfer, these T cells induced diabetes, indicating their pathogenic potential.

At the same time we examined the response of NOD mice to immunization with insulin or insulin peptides, assaying by ELISPOTs (Mohan et al., 2010, 2011). We found no CD4 T cell response when NOD mice were immunized with insulin protein, indicating a level of

unresponsiveness or “tolerance”. In contrast, immunization with B:9–23 peptide generated a strong CD4 T cell response to the peptide, but not to insulin. Thus, the T cells that arose after B:9–23 immunization exquisitely exhibited type B reactivity. In very pointed experiments, we immunized the strain of mice developed by the Eisenbarth group in Denver in which the expressed insulin had a mutation at tyrosine 16 of the B:9–23 residue, a critical residue that when mutated will abolish all T cell responses. Such mice now responded to insulin immunization, a very persuasive finding, indicating that the presence or absence of type A reactivity depended on expression of insulin in the thymus

In brief, the type A insulin-reactive T cells were negatively selected in the thymus, with only a small proportion escaping to enter the peripheral T cell compartment. Furthermore, the type A T cells that were found in the periphery did not react strongly to insulin. By contrast, type B insulin-reactive T cells escaped negative selection in the thymus and participated in the development of disease in NOD mice.

The explanation for the type A and B pMHC lies in register shifting of the B:9–23 peptide in the binding groove of I-A^{g7} (Mohan et al., 2011) (Table 1). (To note, register shifting has been documented with model proteins, particularly ovalbumin, and in the experimental autoimmune encephalitis response to myelin-derived proteins where some registers were associated with disease induction. Different peptide registers dramatically affected the development of encephalitic response) The B:9–23 peptide. binds to I-A^{g7} in two overlapping registers; the first, register 1, includes residues 12–20 and is the one presented after peptide but not after insulin processing. The second register, register 2, includes the residues 13–21, expressed only after insulin processing. Convincing data was obtained by examining presentation of either I-A^{g7} molecules that contained the various segments attached covalently; or soluble peptides containing various segments of the B:9–23 peptide. One particular register is preferentially presented as a result of the protein processing, whereas the other register arise only from the loading of exogenous peptide.

The register 1 (type B 12–20), and register 2 (type A 13–21) peptides have very different binding properties to I-A^{g7}. The 12–20 register binds weakly and generates unstable pMHC complexes having a fast dissociation rate, which increases in the presence of H2-DM. The 13–21 register peptide shows stronger binding and generates a more stable pMHC complex minimally affected by H2-DM. In the late vesicular compartments of APCs, where insulin is taken up, the favored register from the B:9–23 peptide becomes the 13–21 segment, while the unstable 12–20 register is eliminated. Exogenous peptide, on the other hand, binds at the cell surface or in a recycling compartment in both registers, and although the interaction of the type B register with the MHC is short lived, the interaction is long enough to facilitate priming of type B T cells. Of note is that the B:9–23 segment may give rise to T cells recognizing other segments of the peptide, such as a third register (14–22) that is reported to bind very weakly to I-$A^{g7,}$ and it can also provide an MHC class I-binding peptide epitope recognized by $CD8^+$ T cells.

The key to presentation of different epitopes lies in the islet APC

The insulin peptides that generated the type B, reg 1:12–20 pMHC, were presented by the APC that normally reside in the islets of Langerhans. Islets normally contain APCs that are monocyte/macrophage derived, long lived, found tightly adherent to the intraislet vessels, and extend dendrites into the vessel lumen (reviewed in Calderon and Unanue, 2012). The intraislet APC are the reasons for the migration and localization of diabetogenic T cells into the islets during diabetes. Islet APC constitutively capture insulin granules released from the β cells, in the absence of islet inflammation or cell death. The issue to note is that the granules also contain fragments of the insulin B-chain, which bypass the normal secretory

process. The islet APCs present their pMHC to both the type A and B insulin-reactive T cells. In sum, it is the local APC that sets the tone for selection, activation and local attraction of an important set of autoimmune T cells.

Are type B T cells important in other autoimmunities? In many endocrine organs, the cellular basis for the presentation of type B pMHC is present. Hormones, akin to the example with insulin, are synthesized first as a prohormone, in an inactive state that requires proteolysis to generate the active and mature molecule. During its maturation and assembly, catabolic byproducts of the hormone are generated. One example is autoimmune thyroiditis: the thyroid follicle is surrounded normally by DCs. Moreover, anti-thyroglobulin CD4 T cells with reactivity compatible with type B, i.e. peptide positive but unreactive to the proteins, have been identified (cited in Mohan and Unanue, 2012).

Comments

Free peptides or denatured proteins are presented by APCs through an antigen-presentation pathway that is free from the restrictions imposed when peptides are selected from the processing of proteins in late vesicular compartments. This presentation of peptides results in unique and diverse pMHC that are eliminated in the case of protein processing in the APC. The result is the appearance in the APC of the unique pMHC that select for the non-conventional type B CD4 T cells. As we discussed above, these unconventional T cells have been seen before; take the examples discussed on the response to denatured autologous gamma globulin made years ago (McCluskey et al., 1962), and on “cryptic” epitopes (Mohan and Unanue, 2012) which unfortunately were not followed and were not explained. These unconventional peptide-specific T cells are normally found in the response of mice to conventional protein antigens (Mohan and Unanue, 2012) and have also been identified but not studied in humans with autoimmune diseases. Our finding that the type B T cells form a major part of the response to insulin, the major autoantigen in the NOD diabetic mouse, and that the mechanisms for presentation of the insulin peptides have been identified, places these findings on a solid base for future investigations.

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Highlights

- Antigen presenting cells process exogenous peptides differently from proteins
- Many non-conventional CD4 T cells uniquely recognize exogenous peptides.
- In autoimmune diabetes T cells recognize peptides of insulin in islets of Langerhans
- Abundant anti-insulin T cells in NOD mice recognize only exogenous insulin peptide
- Register shift explains differences among anti-insulin T cells in NOD mice

Table 1

Immune Response to Insulin

	Antigen Presentation Assays		ELISPOT Assays	
Antigen Y16A	**Type B T Cells (Reg 1: 12–20)**	**Type A T Cells (Reg 2: 13–21)**	**NOD**	**B:**
Insulin	Neg	+	Neg	+
B: 9–23 (SHLVEALYLVCGERG)	+	+	+	+
Reg 1: 12–20 (VEALYLVCG)	+	Neg	+	+
Reg 2: 13–21 (EALYLVCGE)	Neg	+	Neg	+
14–21 (ALYLVCGER)	Neg	Neg	Neg	Neg

The table summarizes two different sets of experiments. Columns 2 and 3 summarized antigen presentation assays of the two sets of insulin reactive T cells when tested against the antigens shown in column 1, which include insulin and the indicated peptides derived from the B chain. Columns 4 and 5 summarize results of CD4 response in mice immunized with the indicated antigens, testing by ELISPOTs seven days later. ELISPOT assays were done in regular NOD mice or NOD mice expressing a single insulin protein, but with a mutation in the B chain (Tyr16 Ala).

The antigens examined included insulin and the indicated peptides from the B chain. Peptides were presented either covalently bound to I-A^{g7} in the C3.g7 line; or as soluble peptides to DC. Results summarize experiments reported in refs. 2 and 3.