Determinant Capture as a Possible Mechanism of Protection Afforded by Major Histocompatibility Complex Class II Molecules in Autoimmune Disease

By Hongkui Deng,* Raymond Apple,* Michael Clare-Salzler,† Sylvie Trembleau, ‡ Diane Mathis, § Luciano Adorini, ¶ and Eli Sercarz*

From the *Department of Microbiology and Molecular Genetics, and †Department of Medicine (Endocrinology), University of California, Los Angeles, Los Angeles, California 90024-1489; ‡Laboratoire de Genetique Moleculaire des Eucaryotes du Centre National de la Recherche Scientifique, 67085 Strasbourg Cedex, France; and ¶Roche Milano Ricerche, I-20132 Milan, Italy

Summary

How peptide-major histocompatibility complex (MHC) class II complexes are naturally generated is still unknown, but accumulating evidence suggests that unfolding proteins or long peptides can become bound to class II molecules at the dominant determinant before proteolytic cleavage. We have compared the immunogenicity of hen egg-white lysozyme (HEL) in nonobese diabetic (NOD), (NOD x BALB/c)F1, and Eα transgenic NOD mice. We find that a response to the subdominant αNOD determinant disappears upon introduction of an Eα molecule, and is restored when scission of HEL separates this determinant from its adjoining, competitively dominant, Eα-restricted determinant. This suggests that the Eα molecule binds and protects its dominant determinant on a long peptide while captured neighboring determinants are lost during proteolysis. These results provide clear evidence for "determinant capture" as a mechanism of determinant selection during antigen processing and a possible explanation for MHC-protective effects in insulin-dependent diabetes mellitus.

The nature of the actual binding event between an antigenic derivative and class II molecules is still unknown. Peptides eluted from class II are longer than those from class I molecules, mostly 13-22-mer, suggesting that many amino acid residues can protrude from both sides of class II molecules (1, 2). Sette et al. (3) demonstrated that even full-length protein antigens, provided they were unfolded, could directly bind to class II molecules. Interestingly, the unfolded antigens bound preferentially to one of the two class II MHC molecules according to the presence of appropriate binding sites on the antigen molecules.

The possibility thus arises that even local unfolding of a protein molecule, revealing some residues previously inaccessible for binding to class II MHC molecules is sufficient to permit protein binding (4). Proteolytic degradation would follow, leaving a bound peptide with most outlying and flanking residues trimmed. Upon unfolding, the first, most available, and high-affinity binding region on the antigen would preferentially bind to class II molecules. According to this scenario, these protein sequences would be protected from proteolysis by the MHC molecule and could later become the immunodominant T cell determinant (5-7). This mechanism predicts that a single, or a small number of determinants, would emerge as competitively favored over other determinants up- or downstream on the antigen sequence. Furthermore, in binding via the dominant determinant, the mouse class II E molecule for example, would concomitantly capture other A- (or E-) restricted determinants present on the bound protein, reducing the probability that the captured, less dominant determinants could later become involved in productive formation of peptide-class II ligands. Competition between E and A molecules for antigen binding would otherwise only come about with promiscuous peptide determinants (8, 9) binding to both MHC molecules. This general model of determinant capture would readily explain, at a single stroke, many cases of dominance, as well as the existence of cryptic antigenic determinants, that can induce responses but only in the absence of other determinants (10, 11). Likewise, evidence for determinant capture would strongly support a model in which longer peptides regularly bind to class II molecules.

One relevant aspect of determinant capture is its extension to the mechanism of protection provided by added MHC molecules in autoimmune diseases such as insulin-dependent diabeti...
Materials and Methods

**Animals.** Mice were purchased from the Jackson Laboratory (Bar Harbor, ME) or bred in our facility. NOD-E\textsubscript{A}\textsubscript{6} transgenic mice, a transgenic line of NOD mice expressing a wild-type copy (Bar Harbor, ME) or bred in our facility. NOD-E\textsubscript{A}\textsubscript{6} transgenic mice were obtained and examined class II DR-DQ haplotypes in Caucasian and other ethnic IDDM patients and matched controls indicate that known disease-protective alleles have been identified at both the DQB1 and DRB1 loci (17-19).

Determinant capture by transgenic MHC molecules could provide an explanation for disease protection. To establish whether or not this was a suitable model for protection in IDDM, we have compared the immunogenicity of hen egg-white lysozyme (HEL) in NOD, (NOD \times BALB/c)\textsubscript{F1}, and E\textsubscript{A}\textsubscript{6} transgenic NOD mice. This work provides the first clear evidence for "determinant capture" and supports a model in which the protective effects are exerted at the moment of antigen processing in the peripheral immune system.

**Antigens.** HEL, three times recrystallized, was obtained from Sigma Chemical Co. (St. Louis, MO), and was purified by chromatography on a weak cation-exchange column of Bio-Rex 70 (Bio-Rad Laboratories, Richmond, CA). CB-HEL Cyanogen bromide-cleaved HEL (CB-HEL): HEL was treated with a 100-molar excess of resublimated cyanogen bromide in 70% formic acid for 24 h at room temperature and twice lyophilized. Cleavage at residues 12 and 105 was confirmed after reduction of disulfide bonds in CB-HEL as described (Ametani, A., A. Sette, and E. E. Sercarz, manuscript submitted for publication). HEL peptides were synthesized through use of a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA). The peptides were purified by reversed-phase HPLC and showed correct amino acid ratios upon hydrolysis in 6N HCl. Sequences were confirmed by gas-phase microsequencing. A complete series of 15-mer HEL peptides overlapping by 14 amino acids was synthesized using a modified pin synthesis technique (21) and was purchased from Chiron Mito- topes Pty Ltd. (Clayton, Australia). Peptide yield was estimated as described previously (21). Peptides in PBS were directly added to single wells without further purification to a final concentration of 7 \mu M for T cell proliferation assays as described (21, 22).

**T Cell Proliferation.** Mice were immunized subcutaneously in a hind footpad, usually with 7 nmol HEL emulsified in CFA containing H37Ra mycobacteria (Difco Laboratories Inc., Detroit, MI). 9 d later, popliteal lymph node cells were cultured (5 \times 10\textsuperscript{6} per well) in 96-well plates in HEL-1 serum-free medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM glutamine and the indicated concentrations of antigen. Tuberculin purified protein derivative (PPD; Evans Medical, Horsham, UK) was used as a positive control for each culture at a final concentration of 5 \mu g/ml. Proliferation was measured by addition of 1 \mu Ci of [\textsuperscript{3}H]thymidine for the last 15-18 h of a 5-d culture and the incorporation was assayed by liquid scintillation counting.

**Results**

**T Cell Proliferative Response of NOD Mice to HEL.** To map the HEL determinants that elicit an immune response in NOD mice, a panel of 15-mer HEL peptides overlapping by 14 amino acids was synthesized and used to test proliferative responses in draining lymph node cells from mice primed with 7 nmol of HEL. The results shown are representative of three repeated experiments (Fig. 1). Clearly, there are two responsive regions. The major or dominant response was elicited with peptides 6-20 through 14-28. As we have previously described (22, 23), this identifies a "core" sequence, composed of residues 14-20, that is critical for recognition. A minor response was detected with peptides 88-102 through 95-109, indicating a minor, subdominant determinant with a core of 95-102. (We will refer to determinants by their core sequences in the rest of this paper.) These two HEL determinants are recognized in association with I-\textsuperscript{A}\textsubscript{6}, since this is the only MHC class II molecule expressed by NOD mice. Previous studies had identified the HEL 14-20 determinant response in the H-\textsuperscript{A}\textsubscript{4} haplotype (Apple, R., H. Deng, A. Miller, E. Sercarz, and J. Cogswell, manuscript submitted for publica-

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1 Abbreviations used in this paper: CB-HEL, cyanogen bromide-cleaved hen egg-white lysozyme; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic.
To confirm the loss of reactivity to the NOD subdominant determinant 95–102 in F1 mice, we immunized F1 and NOD mice with HEL at varying doses and compared their response patterns. Fig. 3 shows that the 95–102 determinant response can still be detected in NOD mice after low-dose immunization (1.4 nmol) (stimulation index = 6.6), while the F1 mouse displays no 95–102 reactivity, even when 7 nmol of HEL was used for immunization (Fig. 2).

Cyanogen Bromide Cleavage of HEL Restores the Response to 95–102 in F1 Mice. The loss of response to determinant 95–102 in the (NOD × BALB/c)F1 mice could be attributed to a number of factors such as I-E-mediated suppression, effects on antigen processing resulting in the decreased formation of HEL 95–102/A~NOD complexes, or absence from the peripheral T cell repertoire of cells capable of interacting with HEL 95–102/A~NOD after negative selection on I-E self-peptide complexes. One way to test these possibilities directly was provided by the convenient positioning of methionine at residue 105 in HEL. Thus, direct treatment of HEL with cyanogen bromide in 70% formic acid results in scission at Met-105, as well as Met-12, creating flexibility and increased determinant availability at the new termini, although the four disulfide bonds of the derivative (equaling CB-HEL) maintain the overall molecular integrity. Therefore, (NOD × BALB/c)F1 mice were immunized with CB-HEL: as seen in Fig. 4, the response to determinant 95–102 was thereby reestablished, presumably because the scission in the molecule splits apart the determinants 95–102 and 108–116, obviating any determinant capture in which 95–102 responses.
Figure 5. The response to HEL 95-102 can be produced in F1 mice by direct immunization with the peptide. Five representative F1 mice were immunized with 7 nmol of HEL 91-105 in CFA. LNC from these five individual mice were assayed in vitro with peptide 91-105. Each curve represents values from one individual mouse. The backgrounds were subtracted; they ranged from 1,500 to 8,000 cpm.

Discussion

The T cell proliferative response to HEL in the NOD mouse is composed of a very dominant response to an A\textsuperscript{NOD} restricted determinant with a core of 14–20 and a subdominant response centered on residues 95–102. The introduction of an E\textsuperscript{d} molecule, known to exert a dominant role in mice of the H-2\textsuperscript{d} haplotype by binding 108–116, prevented a response to the A\textsuperscript{NOD} binding determinant with the core of 95–102, located upstream on the HEL molecule, a clear example of competitive determinant capture. Determinant capture occurred only if the capturing and the captured determinant were adjoining on HEL. Thus, scission of the bond at Met-105-Asn-106 in creating CB-HEL leaves it as a single entity with its four disulfide bonds intact, but prevents determinant capture and reinstates the response to 95–102. As a matter of fact, presumably the enhanced mobility and availability of 95–102, after cleavage of the 105M-106N bond by cyanogen bromide, led to a more intense response to this determinant. We assume that CB-HEL undergoes reduction

Figure 6. The HEL response pattern is not altered in E\textsuperscript{d} transgenic NOD mice. (A) Five NOD and (B) five NOD-E\textsuperscript{d}16 mice were immunized with 7 nmol per mouse of HEL in CFA. The draining lymph nodes from individual mice were removed 9 d after immunization, and tested for in vitro reactivity against a panel of 15 overlapping HEL peptides. Proliferation was measured on the third day of culture by [3H]thymidine incorporation. Results are expressed as mean counts per minute of triplicates from five mice per group. Only peptides to which a response was raised are shown. (——) 1-18; (··· – –) 8-29; (··· – –) 12-29; (··· – –) 25-43; (··· – –) 94-110; (··· – –) 101-116. The average medium-only backgrounds were 2,113 (for NOD) and 1,672 (for NOD-E\textsuperscript{d}16) cpm.
of the disulfide bonds and further enzymatic attack which fully detaches 106–116 from 91–105.

For determinants to become involved in competitive capture, it is evident that they must lie on the same molecule: short peptides only able to bind to distinct MHC molecules are not subject to determinant capture. It has been shown that long peptides are capable of binding to MHC molecules, provided that they are unfolded, rendering internal agretopes of the antigen accessible. Previous work (3) had shown that reduced full-length protein molecules (lysozyme, OVA, cytochrome c, transferrin) could also bind to class II molecules. More recently, a series of lysozyme derivatives, either full-length or slightly smaller, were shown to directly bind to the E\(\kappa\) molecule, even when only two peptide bonds were cleaved within the HEL molecule as in CB-HEL (Ametani et al., manuscript submitted for publication). Jensen (28) also has shown that native protein antigens, provided that they are reduced/unfolded or under acidification, can bind to MHC molecules.

Our results show that in F1 mice, T cells specific for ANOD complexed to HEL determinant core 95–102 are no longer activated after HEL immunization. If determinant-size peptides were created initially from a protein molecule before MHC binding, it should never arise that E\(\kappa\) and ANOD-restricted determinants would actually compete, because the determinants would lie on separate structures. These determinants happen to be directly in apposition on the native molecule and thereby would be unlikely to be separated by an early random cleavage. Presumably, increasing the distance between the capturing determinant and its target may allow the determinants to become separated by intermediary cleavage or trimming. During the trimming phase, large peptides may be created by endopeptidase cleavage as well as small ones, and the former may get a second chance to bind to MHC molecules.

There is some other evidence supporting the determinant capture model. In vivo, a limited set of processing products (2–15 kD) are bound by class II molecules, as demonstrated by direct detection with a radiolabeled antigen (29). The implication is that some fragments are not further processed but remain bound to MHC molecules. This is in agreement with the observation that peptides as large as 12 kD could be eluted from class II molecules (30). It also has been demonstrated that immunodominance is exerted through intramolecular competition between covalently linked T cell determinants for binding to MHC class II molecules of different isotypes (31, 32). Nepom (33) has presented a competition model in which a single promiscuous diabetogenic peptide is capable of binding, with high affinity, to a variety of class II molecules. We believe that this model involving a single peptide is too restrictive to completely explain the breadth of protective effects afforded by a heterogeneous population of class II molecules encoded at multiple loci.

Our results provide evidence for determinant capture as a mechanism of determinant selection during antigen processing in (NOD × BALB/c)F\(_1\) mice. This mechanism offers insights into the protective effect of I-E or I-A\(^\*\) molecules on the incidence of IDDM in the NOD mouse, as well as for the protective effect of certain DQB1 and DRB1-encoded alleles in human IDDM (17–19). If HEL had been the major autoantigen in IDDM, evidence for HEL determinant capture by E\(\kappa\) molecules in NOD-E\(_{\kappa}\)16 transgenic mice would have easily provided a mechanism for protection. This possibility obviously remains to be tested using reasonable diabetogenic candidates.

In trying to understand the protection afforded by the class II transgenes, two phenomena have lacked an explanation. One is that very different class II MHC molecules can overcome IDDM, including E\(\kappa\)E\(\kappa\) and A\(_2\)A\(_2\). A second derives from the observation that pancreatic biopsies from A\(_k\) transgenic mice show islets mostly free from insulitis but a few with a mild degree of infiltration (13). This indicates that the mechanism of protection is "leaky." Such an incomplete degree of protection has also been reported by Miyazaki et al. (34). These two protection features can be easily explained by the determinant capture model. First, any different MHC molecule may act as a competitive restriction element. However, it is evident that two equally dominant determinants, A\(_2\)A\(_2\)NO\(_D\)/14–20 and E\(\kappa\)E\(\kappa\)/108–116 can concomitantly induce specific T cells. It appears that determinant capture is more likely to occur when there is a disparity in the affinity of the competing determinants along a multideterminant molecule for their different MHC binding sites. Second, an incomplete degree of protection is an inherent feature of the determinant capture model which is dependent on the quantity of a given antigen, the relationship between the binding constants of the competing determinants for the MHC molecule, and the vagaries of antigen processing.
References