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Role of APC in the Selection of Immunodominant T Cell Epitopes

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Following antigenic challenge, MHC-restricted T cell responses are directed against a few dominant antigenic epitopes. Here, evidence is provided demonstrating the importance of APC in modulating the hierarchy of MHC class II-restricted T cell responses. Biochemical analysis of class II:peptide complexes in B cells revealed the presentation of a hierarchy of peptides derived from the Ig self Ag. Functional studies of \( \kappa \) peptide:class II complexes from these cells indicated that nearly 20-fold more of an immunodominant epitope derived from \( \kappa \) L chains was bound to class II DR4 compared with a subdominant epitope from this same Ag. In vivo, T cell responses were preferentially directed against the dominant \( \kappa \) epitope as shown using Ig-primed DR4 transgenic mice. The bias in \( \kappa \) epitope presentation was not linked to differences in class II: \( \kappa \) peptide-binding affinity or epitope editing by HLA-DM. Rather, changes in native Ag structure were found to disrupt presentation of the immunodominant but not the subdominant \( \kappa \) epitope; Ag refolding restored \( \kappa \) epitope presentation. Thus, Ag tertiary conformation along with processing reactions within APC contribute to the selective presentation of a hierarchy of epitopes by MHC class II molecules. The Journal of Immunology, 1999, 163: 6413–6423.

Protein Ags typically contain multiple epitopes capable of binding MHC class II molecules, yet T cell responses are limited to only a small number of these determinants. The ability of the immune system to regulate and focus T cell responses to a select number of epitopes is termed immunodominance (1). The peptide specificities of a T cell response can be classified into a hierarchy of three categories: dominant, subdominant, and cryptic responses. Following in vivo immunization with Ag, bulk T cell responses are directed against a handful of dominant epitopes. These dominant peptides efficiently elicit recall responses in animals primed with whole Ag. In contrast, cryptic peptides cannot restimulate T cells following a primary immunization with native proteins. Cryptic peptides are, however, immunogenic when directly administered in vivo. An intermediate or minimal T cell response corresponds to a subdominant epitope. T cell responses to these epitopes can be recalled with native protein, although at generally higher concentrations than a dominant determinant, and the subdominant peptides themselves are immunogenic.

A number of different mechanisms have been suggested to explain the phenomenon of immunodominance with potentially both APC and T cells playing key roles (1–3). The hierarchy of dominant epitopes displayed on APC may reflect the differential binding of distinct peptides to MHC class II alleles, such that epitopes with the highest affinity are preferentially presented (2, 4). Alternatively, in vivo peptides bound to class II proteins may be selectively edited and exchanged, as has been observed using purified peptide:class II complexes and HLA-DM (5–8). Processing reactions within an APC may also influence immunodominance, since gross changes in Ag structure may modulate the efficiency of an epitope’s presentation (9–11). Biochemical studies have revealed abundant levels of an immunodominant peptide associated with class II complexes isolated from APC (12), yet whether APC directly regulate the hierarchy of presentation for subdominant and cryptic epitopes remains less clear. In fact, at least one cryptic epitope has been identified among the major peptides displayed by murine class II allele on APC (13), thus suggesting T cells rather than APC regulate crypticity and immunodominance. Presumably, both the number of peptide:class II complexes displayed on APC (14, 15) along with the strength of TCR:ligand binding interaction (16) regulate T cell responses. In vivo studies suggest that TCR affinity for distinct peptide:MHC class II complexes may modulate immunodominance (17). Additional constraints guiding immunodominance at the level of the T cell include limitations in reactive T cell precursor frequency and deletions in the TCR repertoire (2, 18).

To investigate the importance of APC and the molecular mechanisms governing epitope selection for immune recognition, natural peptides derived from the Ag human Ig \( \kappa \) were identified bound to class II molecules isolated from a human B lymphoblastoid cell line (B-LCL). Biochemical sequence analysis demonstrated that class II DR4w4 complexes from this APC bound a

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6 Abbreviations used in this paper: B-LCL, B lymphoblastoid cell line; B-HA, biotin-labeled hemagglutinin influenza peptide; DTNB, dithio-bis(2-nitrobenzoic acid); ET, elution time in minutes; I-chain, invariant chain; sDR, soluble HLA-DR; sDM, soluble HLA-DM; TFA, trifluoroacetic acid; HAT, hypoxanthine/aminopterin/thymidine; CHO, Chinese hamster ovary.
hierarchy of κ peptides with one Ig κ epitope (residues 188–203) preferentially loaded vs another κ peptide (residues 145–159). Studies with DR4 transgenic animals established that κ 188–203 was indeed immunodominant whereas κ 145–159 was a subdominant epitope, and therefore supported a role for APC in modulating the hierarchy of T cell responses to this Ag. Quantitative functional assays to measure the number of κ peptide:class II complexes formed within APC demonstrated that the dominant κ 188–203 epitope occupied a higher proportion of the total DR4 molecules. This preferential display of dominant vs subdominant κ epitopes was observed in both human and murine B cells. Each of the κ peptides bound class II DRw4 molecules with a similar affinity to yield stable complexes. Editing or exchange of the κ peptides bound to DR4 was not observed, even in the presence of purified HLA-DM. Together, these results suggest that the hierarchy of κ epitopes presented by DR4 was not modulated by class II:κ peptide binding interactions. Changes in IgG tertiary structure completely reversed the hierarchy of κ epitopes displayed by B cells, whereas Ag refolding restored the immunodominance of κ 188–203. These studies indicate that Ag tertiary structure may prove critical in shaping epitope selection and emphasize the importance of Ag processing in guiding immunodominance.

Materials and Methods

Class II Ag purification

HLA-DR was purified from the B-LCL Priess homozygous for class II DR4w4/Dw53 (DRB1*0401/DRB4*0101). Greater than 90% of the DR Ags expressed by this cell line are DR4w4 (19). Purification of HLA-DR was performed by detergent lysis of cells followed by immunochromatography using the anti-DR mAb LB3.1, with elution of class II molecules at high pH (20, 21). For in vitro studies of peptide binding, class II proteins were purified from DR4w4-transfected Chinese hamster ovary (CHO) cells. Soluble recombiant DR4w4 and HLA-DM, lacking both α and β transmembrane and cytoplasmic domains, were used to examine peptide exchange by DM (5).

Peptide purification and sequence analysis

Peptides associated with purified DR4 were eluted by acid treatment. Two different procedures were employed to separate the eluted peptides from class II proteins; each method resulted in the isolation of nearly an identical profile of peptides as assessed by HPLC. Better quantitative peptide recovery was obtained using the first protocol with ion exchange chromatography. Purified DR peptide complexes were precipitated with 9 vol of cold ethanol for 24 h followed by centrifugation at 10,000 × g for 20 min. The pellet was resuspended in 90% cold ethanol and reprecipitated a total of three times to remove residual detergent, followed by the addition of 200 μl of 0.1% trifluoroacetic acid TFA/20% acetonitrile with incubation for 2 h at 37°C. Samples were centrifuged at 14,000 × g for 5 min to remove insoluble material before peptide purification. Peptides were separated from DR Ags and partially purified by ion exchange chromatography on a Mono S fast protein liquid chromatography (FPLC) column (5/10; Pharmacia, Uppsala, Sweden) in 0.1% TFA/20% acetonitrile. The peptides were eluted with a gradient of 0–1 M NaCl in 0.1% TFA/20% acetonitrile with fractions collected. The purified class II:peptide complexes were also separated and reprecipitated using 10% acetic acid, with peptide isolation following filtration through a 10-kDa membrane (22). Both procedures to separate peptides from class II proteins resulted in nearly identical recoveries of κ peptides. In each case, the isolated peptides were fractionated by reverse phase chromatography on an Aquapore RP300 column (2.1 mm × 100 mm; Applied Biosystems, Foster City, CA) with a linear gradient initialized at 10% acetonitrile/0.1% TFA and progressing to 40% acetonitrile/0.085% TFA after 30 min (flow rate 100 μl/min). N-terminal sequence determination was performed on peptides following HPLC using either an Applied Biosystems 477A or a Porton Industries 2090 protein sequencer and standard procedures. The amount of each peptide in HPLC fractions was estimated based upon amino acid recovery during Edman sequencing (23, 24). Peptide length was confirmed using mass spectral analysis on a Finnigan MAT TSQ 700 equipped with an electrospray source. Synthetic peptides were produced using F-moc technology with an Applied Biosystems Synthesizer, followed by reverse phase HPLC purification and analysis by mass spectroscopy. The migration of synthetic κ peptides on reverse phase HPLC was used to confirm the identity of natural κ peptides eluted from DR4.

Mice and T cell proliferative responses following immunization

B10.M (H-2d; The Jackson Laboratory, Bar Harbor, ME) mice were used to generated transgenic animals expressing chimeric DR4w4 molecules (25). Briefly, the α1 domain (residues 1–85) and β1 domain (residues 1–96) of human DR4w4 were genetically substituted for homologous regions of the murine class II E1α-chain and E1β-chain genes. The resulting chimeric MHC class II proteins retain the peptide-binding specificity of DR4w4, while the putative CD4-binding domain is derived from murine E1. The promoter region for the recombinant class II genes was derived from murine sequences to ensure proper tissue-specific expression within animals. Functional analysis of proliferative responses to Ag was accomplished by foot pad immunization of mice with 200 μg human IgG (Sigma, St. Louis, MO) in 0.1 ml CFA. To establish the immunogenicity of κ peptides, transgenic animals were immunized with 200 μg of each κ peptide in CFA. For all animals, 9 days later, the draining lymph node cells from the popliteal and inguinal nodes were harvested. Lymph node cells were cultured in 0.6% normal mouse serum, RPMI 1640, 1 mM sodium pyruvate, 50 μM 2-ME, 1 mM glutamine, and 10 mM HEPES, and proliferative responses were determined (4 × 103 cells/well with Ag or peptide at 37°C) after 96 h. [3H]Thymidine (6.7 Ci/mol, NEN, Boston, MA) was added (2.5 μCi/well) during the final 12–18 h of this incubation. The results represent the mean experimental (cpm) ± the mean control (no Ag, cpm) ± SEM. All experiments were repeated at least three times in triplicate with the SE <10%.

Generation of B and T cell lines

Human Ig-specific T cell hybridomas were generated by in vitro stimulation of lymph node cells from DR4 transgenic mice (26). Nine days post immunization with 50 μg human IgG in CFA, DR4 transgenic mice were sacrificed, and lymph node cells from these animals were incubated with 10 μM IgG. These cells were fused with the hypoxanthine/aminopterin/thymidine (HAT)-sensitive TCR-negative line BW5147. The resulting T cell hybridomas were selected for drug resistance and tested to determine Ag and MHC restriction. Twenty-seven clonal cell lines were generated from murine clone cell lines were generated from human Ig-specific T cell hybridomas, as well as two additional lines specific for IgG and murine class II proteins. These results suggest that, in DR4 transgenic mice, a substantial amount of the response to human IgG was restricted by human class II DR4. T cell hybridoma function was assessed by cytokine secretion (IL-2) in response to peptide Ag:class II complexes (27). MHC restriction was assessed on APCs/class II complexes with MHC restriction to generated transgenic animals expressing chimeric DR4w4 molecules to ensure proper tissue-specific expression within animals. Functional analysis of proliferative responses to Ag was accomplished by foot pad immunization of mice with 200 μg human IgG (Sigma, St. Louis, MO) in 0.1 ml CFA. To establish the immunogenicity of κ peptides, transgenic animals were immunized with 200 μg of each κ peptide in CFA. For all animals, 9 days later, the draining lymph node cells from the popliteal and inguinal nodes were harvested. Lymph node cells were cultured in 0.6% normal mouse serum, RPMI 1640, 1 mM sodium pyruvate, 50 μM 2-ME, 1 mM glutamine, and 10 mM HEPES, and proliferative responses were determined (4 × 103 cells/well with Ag or peptide at 37°C) after 96 h. [3H]Thymidine (6.7 Ci/mol, NEN, Boston, MA) was added (2.5 μCi/well) during the final 12–18 h of this incubation. The results represent the mean experimental (cpm) ± the mean control (no Ag, cpm) ± SEM. All experiments were repeated at least three times in triplicate with the SE <10%.

To generate murine APCs, LPS splenic B cell blasts from B10.M mice were immortalized by fusion with the HAT-sensitive B cell lymphoma M12.41. The resulting B cell line expressed I-Af/d and I-Ed, as determined by Ab staining and FACs analysis. I-Af expression was comparable to B10.M splenic B cells. These immortalized B cells were transfected by electroporation with the chimeric DR4w4 α and β, with the resulting cloned cell line termed 43.2.DR4. The amount of cell surface DR4 on this murine cell was comparable to the levels expressed on primary B cells derived from the transgenic mice. There was no evidence of DR4 and I-E mispairs in the cell line, as determined by Ab staining and single chain transfectants. Mixed dimers of DR α-chains and specific I-A β-chains have been reported to form at a low frequency in cells dependent upon the presence of a conserved lysine (position 12) in the I-A β-chain (27, 28). No evidence of mixed dimer formation was observed in 43.2 cells transfected with DR α alone, either by staining or functional assays. An independent transfection of 43.2 cells with full-length human DR α also failed to reveal the presence of mixed dimers in functional assays or via Ab capture with DR-specific monoclonals.
Peptide binding and quantitation of class II complexes

A competitive binding assay was used to measure the relative affinity of the κ peptides for DR4 (19). Affinity-purified DR4w4 (10 nM) was incubated with a biotinylated peptide from influenza hemagglutinin, residues 307–319 (B-HA), in the presence or absence of variable amounts of the κ 145–159 or 188–203 peptides. Binding assays in PBS with 1% octylglucoside (pH 6.5 and 5.5) at 37°C were conducted overnight followed by capture of the class II:peptide complexes with the anti-DR α2β2 domain-specific Ab 37.1. The number of B-HA: class II complexes isolated was quantitated using europium-streptavidin and a Wallac Delfia Research Fluorometer (Wallace, Turku, Finland). Peptide binding was saturable in these assays, and the concentration of B-HA used (0.5 nM) was just below the amount required for maximal binding to DR. Purified DR4 was obtained from transfected CHO cells and used as a peptide binding to class II proteins, 1 μg of soluble recombinant DR4w4 (sDR4) was loaded overnight at 37°C with saturating levels of either κ 145–159, κ 188–203, the hemagglutinin peptide 307–319, or a peptide derived from myelin basic protein residues 90–102. Complexes of peptides: sDR4 were then purified by gel filtration chromatography and quantified. Solutions containing 10 nM of each peptide:sDR4 complex were incubated with B-HA (10 μM) at pH 5.0 in the presence or absence of 2 μM recombinant soluble HLA-DM (sDM) as previously described (5). At various time points, aliquots of the reaction were neutralized, flooded with 100 μM unlabeled hemagglutinin peptide 307–319, and transferred to plates coated with the anti-DR specific mAb LB3.1. These plates were developed with europium-streptavidin. Typically 80–90% peptide occupancy is obtained after the overnight loading of sDR4 with peptides. Thus, the small amount of B-HA bound in samples preloaded with the unlabeled hemagglutinin 307–319 or κ peptide is probably due to residual amounts of empty sDR4.

To functionally quantitate κ peptide:DR4 complexes in B-LC1, a modification of the method of Jensen (29) was used to capture isolated complexes for incubation with T cells. For this assay, a standard curve was devised by capturing on plates known amounts of purified peptide-class II complexes. Purified DR4 was obtained from transfected CHO cells and incubated with a high affinity ligand B-HA in the presence or absence of a large excess of the appropriate κ test peptide. These complexes were captured using the anti-DR monoclonal 37.1, and the amount of B-HA bound to class II proteins was monitored using europium streptavidin of known sp. act. The number of κ peptide: class II complexes formed in this competition assay could be calculated based upon the reduction in B-HA: class II complexes. Functional analysis of the standardized κ peptide: class II complexes was determined by addition of T cell hybridomas in tissue culture medium overnight followed by quantitation of cell activation via IL-2 production. A standard curve linking number of peptide: class II complexes with T cell activation could then be obtained. A similar procedure was utilized to determine the number of κ peptide: class II complexes present from APC such as Pries. Total cell class II complexes were isolated using the Ab 37.1 from APC solubilized in 1% octylglucoside/PBS with 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μg/ml chymostatin, and 1000 U/ml DNase at pH 7.2. T cell hybridomas were added to the washed and captured class II complexes with activation monitored as IL-2 production. The standard curve of T cell activation obtained with each hybridoma and known quantities of κ peptide: class II complexes was used to calculate the molar amount of each κ peptide: class II complex formed in cells. Control studies demonstrated that Ab capture of class II complexes did not perturb T cell responsiveness. To calculate the percentage of class II complexes containing each κ epitope, the total number of each peptide: DR4 complex was divided by the total number of DR complexes per cell and then multiplied by 100. The total number of DR molecules per APC was quantitated using an ELISA, and the capture Ab 37.1, along with a secondary class II DR-specific Ab L243, was labeled with biotin and europium-streptavidin.

IL-2 detection

T cell activation was detected by monitoring cell culture supernatants over a range of concentrations for IL-2. Cytokine levels were detected using an ELISA for murine IL-2, or via proliferation of IL-2-dependent HT-2 cells as monitored by [3H]thymidine incorporation. The ELISA protocol entailed capture of murine IL-2, or via proliferation of IL-2-dependent HT-2 cells, as monitored by [3H]thymidine incorporation. The ELISA protocol entailed capture of murine IL-2 from cell culture supernatants (1 h) on EIA/LB3.1 plates. The plates were precoated with 2 μg/ml rat anti-mouse IL-2-specific mAb (PharMingen, San Diego, CA) in 50 mM Tris (pH 9.6). The plates were washed with 0.05% Tween 20 and 0.1% PBS/DTNB, and the modified peptide was resuspended in water before use. Reduction and alkylation of the peptide were confirmed with DTNB, dithio-bis(2-nitrobenzoic acid), revealed that >98% of the κ SH groups within IgG were reduced and alkylated with these procedures. The synthetic κ 188–203 peptide was reduced and alkylated by incubation in 5 M urea/0.1 M Tris-HCl (pH 8.0) with 25 mM DTT for 1 h at room temperature, followed by the addition of 25 mM iodoacetic acid for 1 h. The reduced and alkylated peptide migrated as a single peak on HPLC and was purified on a reverse phase C18 column using an acetonitrile/H2O/TFA gradient, with the peptide containing fractions concentrated to dryness using a speed-vac. Reduction and alkylation of the peptide were confirmed with DTNB, and the modified peptide was resuspended in water before use. Preparations of reduced and alkylated IgG or the modified κ peptide were nontoxic to APC or T cells.

Results

Identification of a hierarchy of naturally derived peptide epitopes from human Ig bound to HLA class II Ags

Qualitative analysis of the peptides released from class II DR4w4 molecules purified from homozigous B-LC1 reveals a multitude of epitopes, the most abundant of which are derived from endogenous self Ags (23, 24). A set of peptides spanning residues 187–208 of the κ L chain of human Ig are prevalent among the species identified bound to DR4, suggesting that these might represent a dominant epitope derived from an endogenous Ag. HPLC and sequence analysis of the major peptides eluted from DR4 demonstrated that, indeed, the endogenous Ig κ 188–203 peptide was relatively abundant along with epitopes derived from HLA class I and I-chain (Fig. 1 and Table I). In addition, a novel, less abundant κ epitope 145–159 was also detected in association with DR4. For these experiments, class II:peptide complexes were isolated from the Ig κ–B-LC1 Pries, and the eluted peptides were HPLC fractionated, followed by sequencing of the major species by Edman degradation and mass spectroscopy. The κ L chain peptide 145–159 migrates as a single species, whereas a nested set of peptides spanning κ 187–203 were detected. The HPLC retention time of synthetic κ peptides further confirmed the identity of these DR4-eluted κ peptides. Based upon algorithms and molecular modeling (19, 23, 35), the minimal DR4w4 binding epitope for the dominant κ peptides encompasses residues 191–200. Estimates of the relative abundance of these natural κ epitopes bound to DR4 suggest
that κ peptides sharing the core 191–200 were at least 6-fold more abundant than κ 145–159. Thus, in B-LCL, a hierarchy of epitopes derived from an endogenous Ag Ig κ are naturally processed and selected for presentation by DR molecules.

A hierarchy of T cell responses is observed following immunization with IgG

The proliferation of cellular immune responses and T cell reactivity have been classically used to define dominant, subdominant and cryptic epitopes within native Ags. Transgenic mice expressing functional DR4 molecules were immunized with human IgG to determine whether the natural Ig κ peptides identified during biochemical analysis of DR4 complexes function in shaping the hierarchy of T cell responses to this Ag. Human IgG was more immunogenic in DR4 transgenic mice vs nontransgenic H-2f mice, as measured by in vitro restimulation of T cells from these animals (Fig. 2). Only lymph node cells from DR4 transgenics primed with IgG proliferated measurably during in vitro restimulation with κ 188–203. In contrast, lymph node T cells from the Ig-primed DR4 mice proliferated minimally or not at all during challenge with the κ 145–159 peptide. Neither κ peptide recalled proliferative responses in the nontransgenic mice expressing murine class II I-Af, confirming the requirement for DR4-restricted presentation of these natural κ peptides (Fig. 2B). However, I-Af-restricted presentation of human IgG was detected in the nontransgenic animals, suggesting that distinct IgG peptides are displayed by this murine class II allele (Fig. 2). Both κ peptides were moderately immunogenic and elicited equivalent T cell responses following immunization of DR4 transgenic mice (Fig. 3). In the nontransgenic mice, T cell responses following peptide priming with each κ peptide were roughly one-third to one-half that observed in the DR4 transgenic animals, suggesting that these epitopes within native IgG may be cryptic for I-Af (data not shown). The immunogenicity of these κ constant region peptides in DR4 transgenic animals was consistent with the minimal sequence homology observed for these

Table 1. Identification of peptides bound to HLA-DR4

<table>
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<th>ET (min)</th>
<th>Sequence</th>
<th>Massa</th>
<th>Protein</th>
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<td>LSWSWTAADTAQITQ</td>
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<td>Bw62 130–144b</td>
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<td>ND</td>
<td>Bw62 129–144b</td>
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<td>VTDFVRFDSDAASQRMEP (±O)</td>
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<td>A2 30–47bc</td>
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<tr>
<td>18</td>
<td>VDDTFVRFDSDAASPR</td>
<td>ND</td>
<td>Cw9 28–44b</td>
</tr>
<tr>
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<td>VDDTVRFDSDAASQR</td>
<td>ND</td>
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<td>2334</td>
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<td>1787</td>
<td>Ig κ 188–203a</td>
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a Peptide mass (Da) as found by mass spectroscopy. ND, not determined.

b Peptides previously identified in Ref. 23 including those derived from HLA-Bw62, -Cw9, -A2 alleles.

c Previously identified in Ref. 24.

d Sequence ambiguity detected as denoted by the symbol X.
human and murine Ig \( \kappa \) epitopes (underlined residues are conserved): \( \kappa \) 145–159 human, KYQWKVDALQGNS; murine, NYVKWKGQRQGQV; \( \kappa \) 188–203 human, KHKVYACEVTHQGLSS; murine, RHNSYTCEATHKTSTS. Together, these results suggested that Ig \( \kappa \) 188–203 was an immunodominant epitope whereas the \( \kappa \) 145–159 peptide may represent a cryptic or subdominant epitope in IgG-primed DR4 transgenic mice.

### T cell specificity for \( \kappa \)-derived peptides

Biochemical analysis indicated that \( \kappa \) 145–159 represents a natural subdominant epitope of human IgG. Yet, we were unable to detect T cell responses to this epitope using lymph node cells from transgenic mice immunized with native IgG, a result more typically associated with cryptic antigenic epitopes. T cell priming in vivo is dependent upon adequate expression of DR4\( \kappa \) 145–159 complexes on APC, and potentially only a very small number of T cells recognizing these DR molecules may have been activated during the initial immunization with exogenous IgG. To investigate this possibility and to examine low frequency T cell populations elicited in the response to human IgG, T cell hybridomas were produced from immunized DR4 transgenic mice. A panel of 27 T cell hybridomas capable of IL-2 secretion in response to IgG and DR4 were identified, with 21 of these cell lines recognizing the \( \kappa \) 188–203 peptide and only one cell line specific for the \( \kappa \) 145–159 peptide. The target peptides recognized by the remaining 5 DR4-restricted hybridomas were not identified; however, these cells potentially may be specific for Ig H chain or \( \kappa \) variable region peptides that bind DR4 (23, 24). For these experiments, DR4 transgenic mice were immunized with human IgG and lymph node cells from the mice restimulated in vitro with Ag, followed by cell fusion with the TCR-negative lymphoma BW5147. Hybridomas were screened for Ag and MHC specificity using murine B cell tumor line 43.2, which had been stably transfected with DR4w4. The specificity of these T cells for DR4 and \( \kappa \) epitopes was also confirmed using multiple human B cell lines expressing DR4 alone or in combination with other DR, DP, and DQ alleles. The low number of T cell hybridomas isolated confirmed that \( \kappa \) 145–159 was indeed a subdominant epitope of IgG and suggested that T cell priming to this epitope was limited.

T cell hybridomas generated using IgG as an Ag were activated by their respective peptides bound to DR4 molecules on the surface of murine or human APC as well as responding to purified class II:peptide complexes. Studies indicate that T cells primed in vivo with native Ag can be used to readily detect and quantitate the presentation of natural epitopes displayed on APC. Proliferative data from two representative IgG-specific T cell hybridomas I/I1.21 recognizing DR4\( \kappa \) 145–159, and I/I2.18 specific for DR4\( \kappa \) 188–203 indicated that these cells exhibited nearly identical dose response curves for their appropriate peptides displayed on a DR4\( ^{+} \) murine B cell line (Fig. 4A). The threshold number of class II:peptide complexes necessary to trigger each T cell was also comparable, as determined using titrating amounts of purified class II Ags loaded with either \( \kappa \) peptide (Fig. 4B). These T cell hybridoma lines recognized both splenic B cells from DR4 transgenic mice as well as \( \lambda ^{+} \) DR4w4 human B-LCL using either the \( \kappa \) peptides or human IgG as a source of exogenous Ag (data not shown). Thus, these T cell hybridomas provide a relative means to compare and quantitate the presentation of each \( \kappa \) epitope by DR4 in a variety of APC.
Quantitation of dominant and subdominant k epitopes using a functional assay

K-specific T cell hybridomas were used to quantitate the number of k peptide:DR4 complexes within APC and to confirm the hierarchy of Ig epitope presentation. Although each T cell hybridoma responded similarly to their respective purified peptides in the context of DR4, a very different profile was observed using these lines and viable APC incubated with native Ag (Fig. 5). Exogenous human IgG is efficiently processed and presented by a DR4 B cell, with measurable numbers of k188–203:DR4 complexes being formed as detected by T cell cytokine production. In contrast, APC required nearly 10-fold more human IgG to comparably stimulate the T cell hybridoma specific for k145–159 (Fig. 5A). Internalization and processing of IgG by APC was required for presentation of both k epitopes, since fixed cells incubated with Ag failed to activate T cells. Presentation of synthetic k peptides was not altered using fixed APC (data not shown). Together, these results indicate that processing of human IgG by B cells results in the preferential presentation of dominant k epitope 188–203, with the peptide k145–159 being a subdominant epitope. Identical results in terms of the hierarchy of k peptides bound to DR4 were obtained using purified k L chains, suggesting that the information guiding epitope processing and presentation remains encoded within the structure of the L chain.

In experiments using murine B cell tumor lines, splenic B cells, or human B-LCL (Igλ+) as a source of APC and exogenous human IgG, the immunodominance of k188–203 was conserved. Although biochemical assays indicated that the hierarchy of k epitope presentation was maintained in B-LCL-producing endogenous Ig, T cell assays were undertaken to directly quantitate the number of functional k peptide:class II DR4 complexes in these cells (Table II). Priess expresses extremely high levels of DR4

FIGURE 4. Functional assessment of Ig k peptide presentation using T cell hybridomas. T cells from human IgG-primed DR4 transgenic mice were immortalized to generate hybridoma cell lines specific for k 145–159 and k 188–203. A, Two hybridoma lines were isolated with nearly identical profiles of activation in response to synthetic versions of their respective k peptide bound to DR4. The T cell hybridoma I/I2.18, which recognizes DR4: k 188–203, and the hybridoma I/I1.21, specific for DR4:k 145–159, were incubated with B cell line 43.2. DR4 and the k peptides 188–203 (■) or 145–159 (●), respectively. IL-2 production was quantitated after 24 h by ELISA. B, Quantitative analysis of peptide:class II DR4 complexes using specific T cell hybridomas. Purified DR4 from CHO cells was incubated with the peptides k 188–203 or k 145–159 overnight at 37°C. These complexes were captured using an anti-DR Ab, followed by addition of T cell hybridomas I/I2.18 (■) or I/I1.21 (●) and measurement of IL-2 secretion. The amount of functional DR4 loaded with each respective k peptide was determined in a competitive binding assay with B-HA, as described in Materials and Methods.

FIGURE 5. The hierarchy of k epitope presentation is conserved among B cells. A dominant T cell epitope, k 188–203, was preferentially displayed by both murine and human B cells using Ig as Ag. A, Activation of k 188–203-specific T cells (■, I/I2.18) was observed over a broad range of human IgG concentrations using the murine B cell 43.2. DR4. Activation of the T cell hybridoma specific for k 145–159 (●, I/I1.21) was observed only using this APC and high concentrations of IgG, confirming this as a subdominant epitope. B, Capture of class II DR4:peptide complexes from the k+ cell Priess indicates that k 188–203 (■) is selectively presented relative to k 145–159 (●). Total class II:peptide complexes from detergent-lysed Priess cells were captured on anti-DR-coated culture plates. Incubation of Ig k-specific T cells I/I1.21 (●) or I/I2.18 (■) with these plates resulted in IL-2 production as measured after 24 h. The amount of T cell activation from a given number of Priess cell equivalents was compared with a standard curve (Fig. 4B) relating T cell responses to a given number of k peptide-class II complexes. This comparison permitted calculations of the total number of each k peptide:class II complex per cell (Table II).
The Journal of Immunology

TABLE II. Quantitation of κ peptide: DR4 complexes in Priess B-LCL*

<table>
<thead>
<tr>
<th>Expt.</th>
<th>DR/Cell</th>
<th>κDR4 Complexes/Cell</th>
<th>% Occupancy</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>κ I</td>
<td>κ II</td>
</tr>
<tr>
<td>1</td>
<td>18 × 10⁶</td>
<td>2.5 × 10⁶</td>
<td>1.0 × 10⁵</td>
</tr>
<tr>
<td>2</td>
<td>13 × 10⁶</td>
<td>1.8 × 10⁶</td>
<td>1.6 × 10⁵</td>
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</table>

* Functional assays were used to quantitate the total number of dominant κ I (residues 188–203) or subdominant κ II (residues 145–159) peptide:DR4 complexes in Priess cells. The number of κ peptide-class II complexes per cell was determined as follows. T cell activation per Priess cell equivalent was calculated from Fig. 5b and converted using the standard curve in Fig. 4b to number of functional κ peptide-class II complexes per cell. The number of DR4 molecules per cell was calculated using a functional κ peptide-class II complexes per cell. Results are shown from two representative experiments.

Figure 6. Ig κ peptides 188–203 and 145–159 bind DR4 with a similar affinity. The in vitro binding affinity of κ 188–203 (○) and 145–158 (□) for purified DR4 molecules was determined in competition assays with a B-HA peptide. B-HA was preloaded with DR4 in the presence or absence of synthetic κ peptides. The amount of B-HA bound was then determined using europium-streptavidin. The half-maximal concentration of each κ peptide necessary to block B-HA binding to DR4 fell between 20–50 nM, and the two distinct κ peptides displayed similar competition curves.

Presentation of the immunodominant κ epitope was dependent upon Ag tertiary structure and processing reactions within APC

Amino acid residues proximal as well as distal from an epitope have been shown to alter the efficiency of presentation, suggesting that Ag primary structure can regulate processing and potentially immunodominance (9–11). Protein tertiary structure may be equally important; thus, the role of Ag conformation and processing in modulating the hierarchy of κ presentation was explored. The dominant κ 188–203 epitope is unique in containing a central cysteine residue (Cys194) required for intrachain disulfide bonding. Structurally, this disulfide linkage might stabilize the κ 188–203 epitope and favor presentation by APC. To determine the importance of IgG conformation and this cysteine residue in κ epitope presentation, human IgG was unfolded by reductive alkylation in the presence of a mild denaturant (30–34). B cells incubated with this unfolded IgG no longer preferentially displayed the κ 188–203 epitope complexed with DR4, as measured by T cell responsiveness (Fig. 8, A and B). By contrast, presentation of the subdominant epitope κ 145–159 was unchanged following perturbation of Ag structure. Presentation of the unfolded IgG to T cells remained dependent upon Ag processing, as shown using fixed APC. Control studies indicated that reduction and alkylation of the synthetic κ 188–203 peptide did not diminish and, if anything, slightly enhanced T cell responses to this epitope presented by APC (Table III). Thus, if the natural κ188–203 epitope was

(>10⁷ molecules per cell), as well as synthesizing abundant amounts of Ig κ. Varying numbers of class II:peptide complexes from Priess cell lysates were immobilized using a DRα2β2 domain-specific mAb before incubation with T cell hybridomas specific for each κ epitope (Fig. 5B). Activation of the T cell specific for κ 188–203 required far fewer total class II complexes (expressed as cell equivalents) in comparison with the κ 145–159 hybridoma. These results demonstrate that there were fewer κ 145–159:DR4 complexes relative to κ 188–203:DR4 formed within Priess cells. Based upon the amount of IL-2 produced by each T cell hybridoma in these studies, we could determine the percentage occupancy for each κ peptide in the Priess-derived DR complexes (Table II). A standard titration curve relating numbers of purified class II:κ peptide complexes with T cell cytokine production had been obtained for each of these hybridomas (Fig. 4B). Nearly 15% of the total DR4 in Priess cells was occupied with Ig κ peptides, and, of this, κ 188–203 occupied roughly 10–20-fold more of the total DR, compared with κ 145–159. Thus, these assays indicate that less than 1% of the total DR4 molecules contained the 145–159 peptide in Priess cells.

Differences in class II:peptide binding affinity were not responsible for the hierarchy of κ epitopes presented by HLA DR4

The above studies strongly suggest that events within APC may guide the selection of immunodominant peptides and thus regulate cellular immune responses in vivo. Differences in the affinity of each of the identified κ peptides for DR4 could potentially account for the preferential loading of class II molecules with the dominant epitope. To test this hypothesis, κ peptide binding to purified DR4w4 was assessed in vitro using a competition assay with a well-characterized DR4w4-binding peptide, biotin-labeled influenza hemagglutinin 307–319 (19, 37). The ability of κ 188–203 and κ 145–159 to compete with biotinylated hemagglutinin 307–319 for binding to DR4 was identical. Inhibition curves for each of the κ peptides were overlapping, indicating that there is little if any detectable difference in the affinities of the two peptides for DR4 (Fig. 6). Inhibition curves had been obtained at pH 6.5 and 5.5 in an attempt to approximate intracellular conditions; however, no difference in the affinity of the peptides for DR4 was detected. These experiments demonstrate that differences in κ peptide:DR4 affinity were not responsible for the hierarchy of Ig κ peptides presented by B cells.

In vitro HLA-DM heterodimers have been shown to facilitate the exchange of unstable or low affinity peptides bound to class II Ags (5, 6, 7) and thus may influence the hierarchy of immunodominant peptides displayed by APC. To examine whether HLA-DM plays a direct role in the selection and stability of Ig κ peptides complexed with DR4, recombinant soluble DR4w4 αβ was preloaded with each of the κ epitopes and incubated with a high affinity competitor peptide biotin-labeled hemagglutinin 307–319, both in the presence or absence of recombinant sDM (Fig. 7). Each of the κ peptides formed stable complexes with DR4, with little peptide exchange mediated by HLA-DM. By contrast, the release from DR4 of a peptide derived from myelin basic protein, 90–102, was significantly enhanced by HLA-DM. Complexes of DR4 preloaded with the hemagglutinin peptide 307–319 were also relatively stable, and HLA-DM did not readily mediate the exchange of this peptide with its biotin-labeled analogue. These results demonstrate that both κ 145–159 and κ 188–203 bind with a high affinity to, and form stable complexes with, DR4. Furthermore, HLA-DM does not facilitate the release or exchange of the subdominant epitope κ 145–159 from DR4.

Presentation of the immunodominant κ epitope was dependent upon Ag tertiary structure and processing reactions within APC

Amino acid residues proximal as well as distal from an epitope have been shown to alter the efficiency of presentation, suggesting that Ag primary structure can regulate processing and potentially immunodominance (9–11). Protein tertiary structure may be equally important; thus, the role of Ag conformation and processing in modulating the hierarchy of κ presentation was explored. The dominant κ 188–203 epitope is unique in containing a central cysteine residue (Cys194) required for intrachain disulfide bonding. Structurally, this disulfide linkage might stabilize the κ 188–203 epitope and favor presentation by APC. To determine the importance of IgG conformation and this cysteine residue in κ epitope presentation, human IgG was unfolded by reductive alkylation in the presence of a mild denaturant (30–34). B cells incubated with this unfolded IgG no longer preferentially displayed the κ 188–203 epitope complexed with DR4, as measured by T cell responsiveness (Fig. 8, A and B). By contrast, presentation of the subdominant epitope κ 145–159 was unchanged following perturbation of Ag structure. Presentation of the unfolded IgG to T cells remained dependent upon Ag processing, as shown using fixed APC. Control studies indicated that reduction and alkylation of the synthetic κ 188–203 peptide did not diminish and, if anything, slightly enhanced T cell responses to this epitope presented by APC (Table III). Thus, if the natural κ188–203 epitope was not...
formed in cells following processing of reduced and alkylated IgG, this peptide should bind with a high affinity to DR4 and be presented to T cells. Furthermore, the hierarchy of κ epitope presentation by B cells could be restored upon refolding of the Ag (Fig. 8C). These observations confirmed that sulfhydryl reduction alone does not influence processing or binding of κ 188–203 to DR4 in B cells. Rather, Ag tertiary structure appears to be key in guiding the processing and preferential presentation of this κ epitope. Treatment of IgG with mild denaturants is required for intrachain disulfide reduction and weakens subunit associations (30–34) as assessed by the appearance of free H and L chains upon aqueous gel exclusion chromatography. A two step process of IgG renaturation can be achieved by dialysis, which allows rapid H and L chain reassociation (32–34). Chromatography of the reduced and alkylated IgG revealed that, after 18 h of dialysis, subunit reassociation was complete. More subtle changes in IgG folding occur during the slow second phase of refolding (32–34); however, complete renaturation of the protein may be hindered by precipitation of the partially folded molecules. Precipitation of the modified IgG was observed during the refolding process, and a lack of complete Ag renaturation may account for the failure to totally restore the efficiency of IgG epitope presentation. Irreversible unfolding of IgG with subunit dissociation can be achieved by boiling the reduced and alkylated Ag. Such harsh treatment of IgG prevented Ag refolding, as assessed by chromatography, as well as diminishing presentation of the κ 188–203 epitope by B cells (data not shown). The extent to which perturbations in IgG folding alter Ag processing at a molecular level remains unclear. However, based upon the selectivity of the T cells used in this study, we predict more than subtle changes in the processing steps giving rise to the κ 188–203 epitope. The T cell hybridoma lines generated in this study can detect a broad range of peptides spanning ε 188–203 presentation did not radically alter display of the subdominant epitope κ 145–159. Thus, the processing of each κ epitope must be independent, and competition between these peptides for DR4 does not determine the hierarchy of presentation by APC.

### Discussion

Processing reactions within APC play a critical role in governing the hierarchy of T cell responses and the selection of immunodominantly and subdominant epitopes. Biochemical as well as cellular approaches were used here to examine mechanisms controlling epitope selection at the level of the APC. Analysis of MHC class II DR4:peptide complexes extracted from a human B-LCL revealed dominant and subdominant epitopes derived from an endogenous Ag, Ig κ L chain. Sequencing and mass spectroscopy indicated that the κ epitope 188–203 was abundant among the Ig peptides eluted from DR4 and present at high levels relative to a second κ peptide spanning residues 145–159. T cell hybridomas specific for each of the κ epitopes were employed to quantitate the number of functional κ peptide:DR4 complexes formed within this APC. With this approach, class II DR4 dimers from B-LCL were found to contain nearly 20-fold more of the κ 188–203 epitope relative to the κ 145–159 epitope. Immunization of HLA DR4 transgenic mice with human IgG revealed that T cell responses were predominantly directed against κ 188–203, thus confirming in vivo the immunodominance of this epitope. By contrast, few of the IgG-primed T cells were specific for the subdominant epitope κ 145–159. Thus, both biochemical and functional assays revealed preferential presentation of one Ig κ epitope vs the other in B cells, emphasizing the importance of APC in the selection of immunodominant epitopes.

APC may regulate immunodominance by a variety of mechanisms, several of which directly influence the abundance of epitopes displayed. The affinity or stability of peptide:class II protein interactions as well as Ag processing may directly dictate the quantity of an epitope displayed on APC. Functional and biochemical studies have demonstrated preferential binding of several immunodominant epitopes to class II proteins, leading to greater numbers of these peptide-class II complexes displayed to T cells (2, 12). Peptide sequencing also revealed the reduced abundance of a minor epitope of hen egg lysozyme, which was attributed to its weaker binding to class II proteins (38). Although the relative affinity of antigenic epitopes for MHC molecules has been correlated...
for DR4. Competition among MHC alleles for peptide ligands could also influence the hierarchy of epitopes presented by APC. However, the immunodominance of Ig κ 188–203 was maintained in both murine and human B cells expressing a variety of class II alleles. Thus, for the Ag IgG κ, there is little evidence that the selection of immunodominant epitopes is solely regulated at the level of peptide:class II binding.

In contrast, evidence was obtained that Ag structure and processing reaction within APC modulate the hierarchy of dominant and subdominant Ig peptides presented to T cells. B cells preferentially displayed immunodominant vs subdominant κ epitopes with native IgG or free κ chains as Ag. Presentation of these epitopes was not observed using native Ag and fixed APC, demonstrating a requirement for intracellular processing in κ epitope presentation. Disruption of Ig L chain tertiary structure radically altered the hierarchy of κ epitopes displayed on class II DR4-expressing APC. Thus with unfolded IgG as Ag, presentation of the dominant epitope κ 188–203 was ablated whereas DR4-restricted display of the subdominant epitope κ 145–159 was unchanged. Ag refolding restored the hierarchy of κ epitope presentation by APC, linking Ag tertiary structure with processing and peptide selection for class II-restricted display. The importance of Ag processing in immunodominance was initially suggested, following demonstrations that immunization with in vitro-processed Ag yielded differences in the hierarchy of T cell responses (10, 11). Similarly, gross changes in Ag structure, such as the insertion of a cryptic epitope into a nonrelated carrier protein, alter immunogenicity (9). Although these studies implicate processing as the key factor in epitope selection, peptide:class II binding interactions or class II allelic competition were not eliminated as potential mechanisms. Additional questions as to the importance of APC and processing in immunodominance were raised by sequencing studies that revealed only a cryptic lysozyme peptide abundantly displayed on murine APC (13). Yet, in the present work, biochemical and functional approaches revealed a hierarchy of κ epitope presentation by APC. Thus, we conclude that APC, along with T cells, play important roles in modulating epitope selection at a molecular level.

Remarkedly, both Ig κ epitopes identified in this study are localized in adjacent antiparallel β strands within the constant region of the Ig L chain. Yet, Ag unfolding prevented presentation of the dominant but not the subdominant κ epitope by B cells. K 188–203, the dominant epitope, contains an invariant cysteine residue critical to disulfide bonding and the folding of all Ig L chains. The presence of this intrachain cysteine bond may facilitate the preferential presentation of this epitope through maintenance of IgG tertiary structure and protection from premature protease digestion in B cells. Denaturation of IgG along with cysteine reduction

with immunogenicity (39, 40), this may not be the only factor influencing peptide-class II complex formation. Cryptic (13) and, here, subdominant peptides have been identified that efficiently bind to class II Ags. Both Ig κ 188–203 and 145–159 bound with a high and comparable affinity to DR4, indicating that differences in peptide binding do not directly modulate the observed hierarchy of κ epitopes presented by B cells. In vivo, class II:peptide interactions are modulated in part by HLA-DM, which potentiates the rate of I-chain peptide release from nascent class II complexes (5–7). HLA-DM may also enhance the exchange of other class II-associated peptides (5, 8); however, a specific role for DM in the selection of immunodominant epitopes remains unclear. Our studies indicate that the inclusion of purified DM in binding assays does not alter the affinity of the subdominant 145–159 κ epitope

Table III. Functional analysis of κ 188-203 peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>T Cell Activation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>κ188-203 (0.1 μM)</td>
<td>663.9 ± 108</td>
</tr>
<tr>
<td>RA κ188-203 (0.1 μM)</td>
<td>1797 ± 511</td>
</tr>
<tr>
<td>κ188-203 (10 μM)</td>
<td>246,997.8 ± 2190</td>
</tr>
<tr>
<td>RA κ188-203 (10 μM)</td>
<td>286,638.4 ± 11,167</td>
</tr>
</tbody>
</table>

*DR4-expressing APC were incubated with variable concentrations of the synthetic peptide κ188-203 as well as reduced and alkylated (RA) κ188-203. Activation of the I/12.18 T cell by peptide-pulsed APC was monitored in a bioassay using proliferation of the cytokine-dependent HT-2 cell line. Data are expressed as mean cpm of tritiated thymidine incorporated by HT-2 cells ± SEM.

FIGURE 8. Effect of Ag conformation on κ epitope selection by B cells. Unfolding of human IgG by denaturing reductive alkylation disrupted presentation of the immunodominant κ epitope without altering the display of the subdominant κ epitope by APC. A. DR4* murine B cells incubated with native human IgG preferentially presented the dominant κ 188–203 (■) epitope vs subdominant κ 145–159 (●) peptide to T cells. B. IgG unfolding and reductive alkylation followed by 18-h dialysis altered the processing and presentation of the κ 188–203 epitope (■) by B cells. The formation and display of κ 145–159:DR4 complexes was not perturbed by changes in Ag conformation (●). C. Extensive dialysis (2–4 days) facilitated IgG refolding and restored the preferential presentation of κ 188–203 by B cells. APC were cultured (3 h) with untreated or treated Ag, washed, and incubated with κ 188–203-specific T cells (I/12.18) or κ 145–159-specific I/11.21 cells. IL-2 production was quantitated using HT-2 cells.
might therefore be predicted to disrupt the presentation of this \( \kappa \) epitope, as was observed. In B cells, disulfide reduction of Ags has been localized to endosomal compartments containing class II proteins as well as dense lysosomes (41). Potentially, endosomal co-localization of class II proteins and Ag reduction in B cells may be critical in facilitating MHC-guided processing and the selection of some immunodominant epitopes (42). Thus, class II DR4 molecules could sequester the dominant 188–203 epitope upon Ag reduction, with this complex of Ag and MHC proteins serving as a template to guide processing. By contrast, presentation of the subdominant \( \kappa 145–159 \) epitope was not altered by Ag unfolding, revealing little requirement for Ag tertiary conformation in the class II-restricted display of this peptide. Additionally, competition between these \( \kappa \) epitopes for binding to class II molecules does not appear critical in determining the hierarchy of peptides displayed, since loss of \( \kappa 188–203 \) presentation did not influence presentation of \( \kappa 145–159 \).

In vivo studies reveal changes in the hierarchy of T cell responses with the development and progression of autoimmune disease (43). The molecular events that perturb epitope selection in these cases remain unknown; however, changes in autoantigen structure and the infiltration of novel APC may prove key. Inflammatory reactions due to viral, bacterial, or physical trauma have been postulated as initiators of autoimmune responses. The release of host/pathogen proteases as well as free radicals during inflammation potentially could alter the structure of autoantigens and influence processing and presentation by APC. In this regard, presentation of an epitope from an interphotoreceptor retinoid binding protein by professional APC requires preprocessing of this self Ag by extracellular proteases from retinal cells (44). The present study carries this observation further to directly demonstrate that disruptions in autoantigen structure can lead to changes in the hierarchy of immunodominant epitopes displayed on APC. Such changes in epitope selection may disrupt self tolerance and potentiate the induction of autoimmune disease.

Acknowledgments

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References