

## INFLUENCES OF ANTIGEN PROCESSING ON THE EXPRESSION OF THE T CELL REPERTOIRE

### Evidence for MHC-specific Hindering Structures on the Products of Processing

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The expressed T cell repertoire to many proteins has been found to be focused onto a limited number of discrete immunodominant sites rather than broadly directed at all segments of an antigen. The phenomenon has also been shown to occur for noneukaryotic proteins, such as influenza hemagglutinin (1), staphylococcal nuclease (2), and  $\beta$ -galactosidase (3), that are unrelated to self proteins of the mouse. There appear to be several factors that influence the expressed repertoire to a particular protein. The most clearly demonstrable influence is the MHC, and this appears to be the basis of *I*r gene effects, where differences in T cell responsiveness to protein antigens occur between different strains of mice. Three major mechanisms have been suggested by which MHC-linked genes may influence immune responsiveness (4). Evidence has been obtained for a determinant selection mechanism by the demonstration of direct binding of immunodominant peptides to class II MHC restriction molecules that can present the peptide but not to class II MHC molecules that do not present the peptide (5, 6). There is also some evidence for MHC-encoded molecules limiting the expressed repertoire of T cell clones during thymic development (hole-in-the-repertoire model) (4), as well as for MHC-linked *I*r gene-controlled responses dominated by active suppression in low responder strains (7).

In this paper, we present evidence for a fourth major mechanism controlling the expressed epitope repertoire and immunodominance involving the specificity of antigen processing and the existence of hindering structures on naturally processed fragments that differentially affect presentation by different MHC molecules. Many complex antigens, in contrast to short peptides, have been shown to require processing by APCs before they can be presented in an immunogenic form in association with class II MHC to T helper cells (8). The molecular mechanisms involved in processing are not fully understood. Processing can be inhibited by fixing APC with paraformaldehyde or by treatment with agents such as chloroquine or ammonium chloride, which raise lysosomal and endosomal pH and interfere with lysosomal and endosomal function, and also with monensin, which inhibits receptor recycling through the Golgi apparatus (8-10). Antigen processing was also inhibited by protease inhibitors such as leupeptin (9-11), suggesting processing involved proteolysis

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of the antigen into smaller fragments. However, some studies suggested that denaturation (12) or unfolding (10) may be all that is necessary for processing of at least some sites, and an unfolded portion of fibrinogen did not appear to require any processing (13).

At the present time, however, little is known about the chemical nature of processed antigen. There is some evidence that the hierarchy of T cell responses to different determinants on an antigen may be influenced by the particular forms of processed antigen that occur. For example, in the hen egg lysozyme (HEL)<sup>1</sup> system, processing differences have been suggested to explain the observations that the choice between two distinct T cell determinants within a 23-amino acid region (T11) depends on their structural context (14). Also, with lysozyme, differential processing of variant forms of the antigen appears to result in the heteroclicity of B6 clones for ring-necked pheasant lysosome (REL) in preference to HEL (15).

In the course of investigating the difference in immunogenicity and antigenicity of whole sperm whale and equine myoglobin and synthetic peptide fragments of these proteins, we found two distinct lines of evidence that the choice of T cell determinants potentially available on complex protein antigens may not only be a function of Ia-peptide interaction but may also be related to processing events. Both lines of evidence point to the existence of MHC-specific hindering structures on naturally processed fragments that are not present on synthetic peptides comprising little more than the minimal antigenic site. First, after immunization with whole equine or sperm whale myoglobin, different peptides became the focus of the response in mice of different MHC haplotypes. However, individual clones were found in myoglobin-immune mice that responded to peptides that did not stimulate a response in the bulk immune lymph node T cell population. Moreover, immunization with the peptides themselves revealed some new reactivities that had not appeared after priming with whole myoglobin. In at least two cases, peptide immune T cells appeared to be poorly crossreactive with whole myoglobin. Therefore, low responsiveness to these epitopes could be explained neither by the lack of an appropriate T cell repertoire nor by failure of the peptide to be presented by the class II MHC molecules of that strain. A second independent line of evidence was the existence of T cell clones whose fine specificity for variant sequences was different when measured with native proteins vs. synthetic peptides. These studies suggest that the structure of the antigen fragment resulting from natural processing is important, because flanking regions outside the epitope may interfere with binding to Ia or to the TCR. This may have important implications for the design of peptide vaccines that, although immunogenic themselves, may generate a T cell response that is poorly reactive to the native molecule.

### Materials and Methods

*Myoglobin.* Equine and sperm whale myoglobin were obtained from Accurate Chemical and Scientific Co. (Hicksville, NY). Sperm whale myoglobin was purified by ion-exchange chromatography by the method of Hapner et al. (16), and the major chromatographic component, IV, was used for all studies.

*Myoglobin Cleavage Fragments.* Apo-myoglobin and CNBr cleavage fragments of equine myoglobin were prepared as previously described (17).

<sup>1</sup> *Abbreviations used in this paper:* HEL, hen egg lysozyme; REL, ring-necked pheasant egg lysozyme.

**Peptide Synthesis.** A nested series of peptides corresponding to region 102-118 of equine and sperm whale myoglobin were prepared on a Vega 250 peptide synthesizer by using the solid-phase method of Merrifield (18) as modified by Corley et al. (19). The standard t-boc/benzyl amino acid protection strategy was used with side chain protection of the following amino acids obtained from Vega Biochemicals (Tucson, AZ): Asp(*O*-benzyl), Glu(*O*-benzyl), His(tosyl), Lys(2-chlorobenzoyloxycarbonyl), Ser(benzyl), and Tyr(2,6-dichlorobenzyl). The extent of the double dicyclohexylcarbodiimide-mediated couplings was monitored using a qualitative ninhydrin test, and recoupling was performed when <99% coupling was observed. Peptides were cleaved from the resin by using the standard high HF method containing 10% *p*-cresol for 40 min at 0°C, washed with ethyl acetate, and extracted with acetic acid before lyophilizing. Peptides were purified to homogeneity by gel filtration on a Biogel P4 column in 9% formic acid and by reverse-phase HPLC described previously (20). Composition was confirmed and concentration was determined by amino acid analysis (kindly performed by Robert Boykins, FDA). The peptides from the sperm whale sequence were described previously (21).

**Mice.** B10.BR (H-2<sup>k</sup>) and B10.D2 (H-2<sup>d</sup>) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B10.S (H-2<sup>s</sup>), B10.A(4R) (I-A<sup>k</sup>, I-E<sup>-</sup>), (B10.S × B10.BR)F<sub>1</sub>, and (BALB/c × A.SW)F<sub>1</sub> (H-2<sup>d</sup> × H-2<sup>s</sup>)F<sub>1</sub> mice were bred in our own facility.

**Lymph Node Proliferation Assays.** Mice were immunized subcutaneously in the tail with 100 µg equine or sperm whale myoglobin in CFA. Alternatively, mice were immunized in the same way with 4 nmol synthetic peptide. 8 d later, draining inguinal and periaortic lymph nodes were removed, and single cell suspensions were prepared (22). Assays were set up with triplicate wells with appropriate antigen in 96-well plates with  $3.5 \times 10^5$  cells/well in complete medium consisting of RPMI 1640 with 44% Eagle's-Hanks' amino acid medium, 10% FCS,  $5 \times 10^{-5}$  M 2-ME, 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco Laboratories). Plates were incubated for 4 d at 37°C in 5% CO<sub>2</sub>, pulsed with 1 µCi of [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA), and harvested 18 h later onto glass fiber paper. [<sup>3</sup>H]thymidine incorporation into DNA was quantitated by liquid scintillation counting. The geometric mean and SEM for each group were determined, and the no antigen background was subtracted to obtain Δ cpm.

**Establishment of Myoglobin-specific Lines and Clones.** Equine myoglobin-specific polyclonal lines were established by the method of Kimoto and Fathman (23) as modified by Matis et al. (24). Five mice were immunized in the footpad with 100 µg equine myoglobin in CFA. 8 d later, draining inguinal and popliteal lymph nodes were removed, and the cell suspension was enriched for T lymphocytes by passage over a nylon wool column. Purified T cells were incubated at  $4 \times 10^6$  cells/well in 24-well plates (No. 3524; Costar, Cambridge, MA) with  $2 \times 10^5$  irradiated syngeneic spleen cells and equine myoglobin at 1 µM in complete tissue culture medium (see above). 4 d later, responding T blasts were purified by Ficoll density gradient centrifugation. The T cells were put in resting culture at  $2 \times 10^5$  cells/well with  $4 \times 10^6$  irradiated spleen cells for 10 d; then  $5 \times 10^6$  irradiated spleen cells plus myoglobin were added to begin another round of stimulation and rest. T cell lines were cloned by limiting dilution after one or two rounds of stimulation and rest. The cloning was carried out 48 h after stimulation in the presence of antigen,  $10^6$  irradiated spleen cells, and 5 U/ml IL-2-containing supernatant from EL4 T lymphoma cells stimulated with mezerein. T cells were cloned at 1 and 0.4 cells/well. Positive wells were expanded after 14 d into 24-well plates with additional antigen, spleen cells, and IL-2. After further expansion, cells were washed, rested, and then stimulated without IL-2 in 2-wk cycles.

**Proliferation Assay.** Cloned T cells ( $10^4/0.2$  ml) in microtiter wells containing various concentrations of antigen with  $5 \times 10^5$  irradiated syngeneic spleen cells as APC were cultured for 4 d; 1 µCi [<sup>3</sup>H]thymidine was added 18 h before harvesting. Results are expressed as geometric mean ± SEM of [<sup>3</sup>H]thymidine incorporation of triplicate wells.

## Results

Previous studies have shown that B10.BR and B10.S mouse strains are both high responders to equine myoglobin (25). Using myoglobin sequence variants, residue

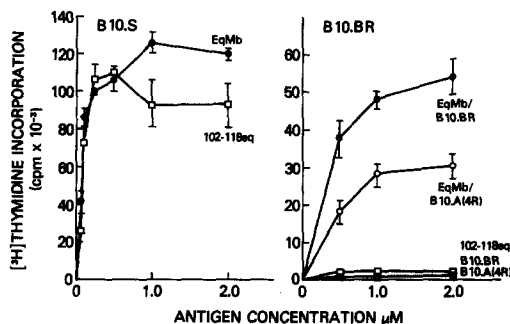


FIGURE 1. Proliferative responses to equine myoglobin-specific short-term polyclonal cell lines from B10.BR and B10.S mice show markedly different reactivity to peptide 102-118. T lymphocytes ( $10^4$ ) were cultured with  $5 \times 10^5$  irradiated syngeneic spleen cells, together with various concentrations of antigen. B10.BR T cells were also incubated with B10.A(4R) spleen cells that express only I-A<sup>k</sup>. Control cultures without antigen from T cells + B10.BR APC gave 1,377 cpm, T cells + B10.A(4R) APC gave 2,514 cpm, and T cells + B10.S APC gave 1,406 cpm. Results are expressed as geometric mean  $\pm$  SEM.

109 was identified as being critical for stimulation of B10.S T cells (26). In contrast, a totally different crossreactivity pattern was observed for B10.BR T cells, as they responded only to equine myoglobin and not to a panel of 16 other myoglobins tested (data not shown). To define more precisely the major determinants recognized by these two strains, synthetic peptides corresponding to different regions of the equine myoglobin sequence were prepared and tested. Results in Fig. 1 show that the peptide 102-118 was able to stimulate equine myoglobin-immune B10.S T cells as effectively as equine myoglobin itself, confirming that this region is immunodominant for B10.S T cells. Because B10.S mice express only I-A<sup>s</sup> and do not produce an I-E molecule, it is likely that this peptide is presented in association with I-A<sup>s</sup>. In contrast to the results seen with B10.S T cells, the 102-118 region does not appear to be stimulatory for B10.BR T cells immune to equine myoglobin. There was little (<5%) or no detectable response to this peptide compared with equine myoglobin in immune lymph node cultures (not shown) or short-term polyclonal cell lines (Fig. 1). Results in Fig. 1 also show that the short-term B10.BR polyclonal line had no alloreactivity to B10.A(4R) cells, which express only I-A<sup>k</sup>. B10.A(4R) spleen cells were able to present equine myoglobin to this line, although not as well as B10.BR spleen cells, indicating that both I-A- and I-E-restricted T cells may be involved in this response.

*Cloning Reveals Equine Myoglobin 102-118-specific B10.BR T Cell Clones of Fine Specificity Similar to Those from B10.S Mice.* A panel of equine myoglobin-specific T cell clones established from B10.S and B10.BR mice were screened for reactivity to the 102-118 peptide. It was found that 16 of 17 B10.S clones responded to this peptide, confirming the immunodominance of this region at the clonal level. These clones are probably I-A<sup>s</sup> restricted, because B10.S mice do not express an I-E molecule. Surprisingly, 3 of 16 equine myoglobin-specific clones from B10.BR mice also responded to the 102-118 peptide. The 102-118-specific B10.BR clones were shown to be I-A<sup>k</sup> restricted, because they responded on B10.A(4R) APC, which express only I-A<sup>k</sup> and not I-E<sup>k</sup> and because their response was blocked by the anti-I-A<sup>k</sup> mAb 10.2.16 but not by the anti-I-E<sup>k</sup> antibody 14.4.4.

It was surprising to find 3 of 16 of the B10.BR clones responding to peptide 102-118, because very little, if any, response is observed to this peptide in the polyclonal population. It was therefore of interest to see if the fine specificity of these clones was the same as that of 102-118-specific clones from B10.S mice, in which this peptide

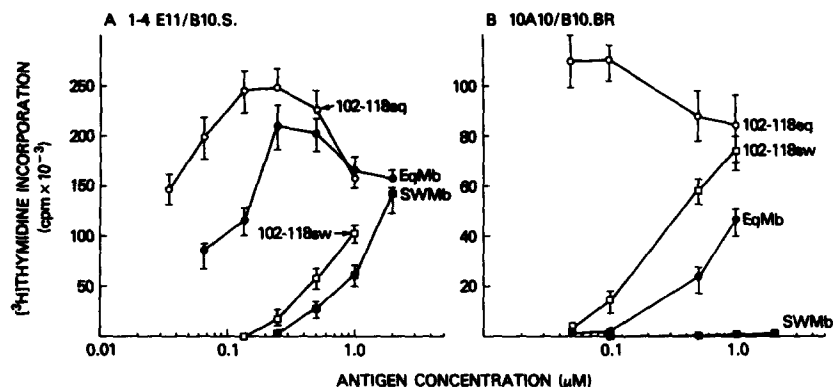


FIGURE 2. Proliferative response of equine myoglobin-specific I-A<sup>s</sup>-restricted clone 1-4E11 (A) and I-A<sup>k</sup>-restricted clone 10A10 (B) to whole equine or sperm whale myoglobin or to synthetic peptides corresponding to region 102-118 of these molecules. Culture conditions and results are as in Fig. 1. Background proliferation was <1,000 cpm for both clones.

is immunodominant. Crossreactivity was assessed for sperm whale myoglobin and for the 102-118 peptide therefrom, which differs from the equine sequence at two residues, 109 and 118. The fine specificity of the B10.S clones showed three main patterns with respect to the 102-118sw peptide. The majority of the clones (10 of 16) showed no responsiveness to either sperm whale myoglobin or 102-118sw and corresponded to the predominant pattern observed in lymph node cultures and short-term lines (26 and unpublished observations). Over one-third (6 of 16) of the clones, however, showed some responsiveness to sperm whale myoglobin or the peptide (Fig. 2 A), but the degree of cross-stimulation varied from clone to clone. The fine specificity pattern of the B10.BR clones, such as 10A10, was of the latter type (Fig. 2 B). All the I-A<sup>k</sup>-restricted clones responded to 102-118sw, although this peptide was less potent than the 102-118eq peptide. Variation in relative responsiveness among the three clones indicated that these were distinct clones. Thus, overall, the B10.BR clones specific for this region showed fine specificity similar to that of a significant fraction of B10.S clones.

For the B10.S clones, the 102-118 peptide was 1.5 to 3 times more potent than whole myoglobin on a molar basis (Fig. 2 A). In contrast, for all the B10.BR clones, the peptide was at least 10 times more potent than whole myoglobin (Fig. 2 B). To investigate whether these observations reflected differential antigen processing of equine myoglobin by B10.BR and B10.S APC, the ability of the same APC, (B10.BR  $\times$  B10.S) $F_1$ , to present whole equine myoglobin to the B10.BR and B10.S clones was assessed. The  $F_1$  APC, which presented equine myoglobin to B10.S clones as well as B10.S APC, did not present equine myoglobin to B10.BR T cell clones any better than B10.BR APC did (Fig. 3). A similar result was found for other B10.BR and B10.S clones as well. Thus, the inefficient presentation by B10.BR APC of this site of equine myoglobin is not improved with the  $F_1$  APC that bear the I-A<sup>s</sup> molecule capable of presenting this region of equine myoglobin more efficiently. We conclude that the poor responsiveness to whole equine myoglobin versus peptide does not appear to be due to MHC-linked processing or the availability of all antigenic frag-

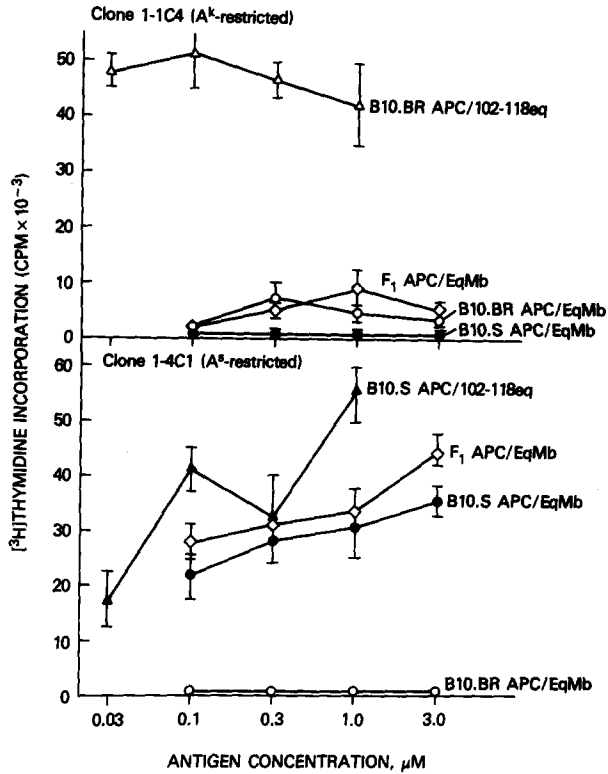


FIGURE 3. Test for an antigen-processing defect in B10.BR vs. B10.S or F<sub>1</sub> APC. Proliferative response of B10.BR T cell clone (1-1C4) and B10.S clone (1-4C1) to equine myoglobin when incubated with APC from B10.BR (○), B10.S (●), and (B10.BR × B10.S)F<sub>1</sub> (◇) mice. Proliferation stimulated by 102-118eq peptide on B10.BR (Δ) or B10.S (▲) APC is shown for comparison. Background proliferation was <1,000 cpm for all clones.

ments in different APC, but is dependent on the MHC molecule used for presentation. The response of B10.S clones to whole myoglobin does not appear to be influenced by the presence of the I-A<sup>k</sup> haplotype in the F<sub>1</sub> macrophages, indicating that it exerts no inhibitory or competitive effects. Also noteworthy, there was no degeneracy in the ability of I-A<sup>k</sup> and I-A<sup>s</sup> to crosspresent equine myoglobin to clones of the other haplotype, although both I-A molecules can present the same site (Fig. 3).

*Response of 102-118eq Immune Lymph Node Cells Reveals a New Repertoire.* Since 3 of 16 B10.BR clones obtained responded to the 102-118 region, it appeared that low responsiveness of the polyclonal B10.BR T cells to this epitope was due neither to an inability of I-A<sup>k</sup> to present this epitope nor to an absence of T cells in the repertoire. To further investigate the ability of B10.BR mice to respond to this region, mice of B10.BR and B10.S strains were immunized with the synthetic 102-118eq peptide in CFA. Surprisingly, the peptide was immunogenic in both strains (Fig. 4). However, while the 102-118eq B10.S immune T cells responded as well to whole equine myoglobin as they did to the peptide, B10.BR T cells responded to the peptide but only very weakly to whole myoglobin. Also, whereas 102-118eq-specific B10.S T cells were only weakly crossreactive to 102-118sw, corresponding to the overall pattern observed in whole equine myoglobin-immune B10.S lymph node cells and most of the T cell clones, 102-118eq-specific B10.BR T cells responded well to both equine and sperm whale peptides. Because the backgrounds of the lymph node proliferation experiments tended to be high, a short-term polyclonal line was established from

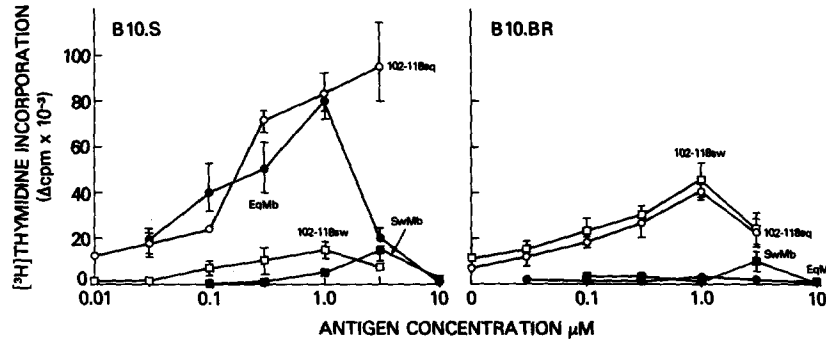


FIGURE 4. Response of 102-118eq-specific lymph node cells. Repertoire revealed on immunization with peptide. Proliferative response of  $4 \times 10^5$  unpurified lymph node cells from B10.S and B10.BR mice immunized with synthetic peptide 102-118 of the equine sequence to various concentrations of whole equine or sperm whale myoglobin or peptides 102-118eq and 102-118sw. Background cultures with medium alone were 19,678 cpm for B10.S cells and 16,897 cpm for B10.BR cells. Results are plotted as geometric mean cpm of triplicate cultures ( $\times/\pm$  geometric SEM). PPD responses for B10.BR cells were 114,126 (1.08) cpm and for B10.S were 162,434 (1.07) cpm.

102-118-immune B10.BR lymph node cells. This line showed similar results to that obtained with the lymph node cultures (Fig. 5). However, as the line was enriched for antigen-specific T cells, the total response was enhanced. The increased sensitivity allowed detection of the response to whole equine myoglobin, but there was still a dramatic difference in the dose response to whole equine myoglobin versus peptide (Fig. 5). The proliferative response of the 102-118eq B10.BR line was fully blocked in the presence of an anti-I-A<sup>k</sup> mAb (10.2.16) and not by an anti-I-E<sup>k</sup> antibody (14.4.4), suggesting that the majority of T cells in this line were I-A<sup>k</sup> restricted (data not shown). We are forced to conclude that I-A<sup>k</sup> can present this peptide and that clones exist in the T cell repertoire specific for this site. The lack of crossreactivity for whole equine myoglobin among peptide 102-118-immune T cells in B10.BR mice suggested an explanation for the low responsiveness to this epitope in equine myoglobin-immune mice (see Discussion).

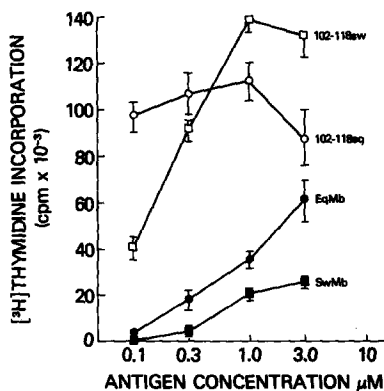


FIGURE 5. Proliferative response of short-term B10.BR anti-102-118eq T cell line to various concentrations of whole equine or sperm whale myoglobin of peptides 102-118eq and 102-118sw. Background cultures with medium alone were 673 cpm.

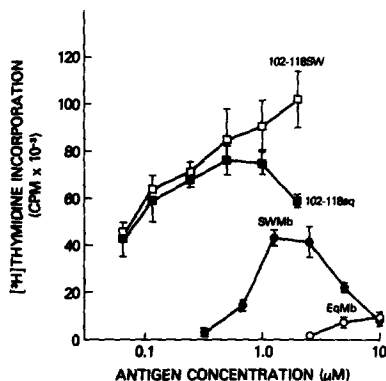


FIGURE 6. Crossreactivity at the peptide but not the protein level. Proliferative response of clone 9.27 to whole equine or sperm whale myoglobin or to synthetic peptides 102-118eq and 102-118sw. Culture conditions and results are as in Fig. 1. Background proliferation was 385 cpm.

*Response of H-2<sup>d</sup> Clone 9.27 to Myoglobin Variant Synthetic Peptides But Not Whole Proteins Provides a Second Line of Evidence.* Previous studies using sequence variants of native myoglobin suggested that the site in native sperm whale myoglobin seen by B10.D2 (H-2<sup>d</sup>)-immune lymph node cells and T cell clones included Glu 109 and possibly His 116 (27). Using a nested series of peptides corresponding to region 102-118 of sperm whale myoglobin, it was subsequently shown that these two residues were indeed included in this immunodominant T cell site recognized in association with I-A<sup>d</sup>. A consensus sequence from 106-118 was required for stimulation of the clones (21). Lack of crossreactivity with equine myoglobin was attributed to the substitution at position 109 of Asp for Glu on the basis of a panel of 15 variant myoglobins (27). This was reconfirmed for clone 9.27 in the present study (data not shown). It was therefore surprising to find that, for clone 9.27, the 102-118 peptide of equine myoglobin was as potent as the sperm whale peptide over a wide dose-response range in contrast to the response to the native proteins (Fig. 6). Other I-A<sup>d</sup>-restricted 102-118-specific clones were examined, and while these all hardly responded to native equine myoglobin, they showed different degrees of responsiveness to the equine peptide, as found with the B10.S clones. For example, clone 30-06 showed no response to the peptide, whereas clone 9.23 responded, but less well than to the sperm

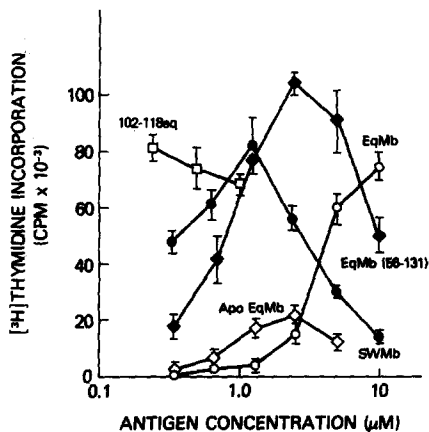


FIGURE 7. Proliferative response of clone 9.27 to equine and sperm whale myoglobin, apo-equine myoglobin, CNBr middle fragment (56-131), and synthetic peptide 102-118eq. Culture conditions and results are as in Fig. 1. Background proliferation was 811 cpm.



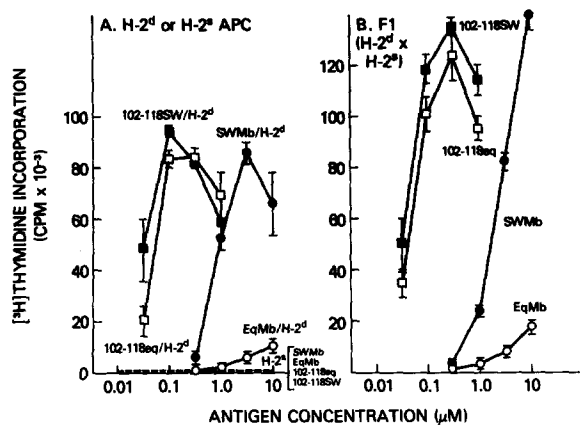


FIGURE 8. Reactivity differences are maintained in the same F<sub>1</sub> APC. Proliferative response of clone 9.27 to various concentrations of antigens when incubated with APC from BALB/c (H-2<sup>d</sup>), A.S.W (H-2<sup>s</sup>) (left), and (BALB/c × A.S.W)F<sub>1</sub> (right) mice. Antigens tested were sperm whale and equine myoglobin and peptides 102-118sw and 102-118eq. Background proliferation was <1,000 cpm for all the medium alone controls.

whale peptide (data not shown). Parenthetically, this confirms and extends the results reported by Cease et al. (21) that the response to this region represents focusing onto an immunodominant site of T cells with a range of fine specificity patterns. However, the fact that many H-2<sup>d</sup> clones do not distinguish the equine and sperm whale 102-118 peptides as they do the native molecule provides a second line of evidence that the processed form of the native molecules is hindered in its presentation or recognition compared with the synthetic peptides.

*Sperm Whale/Equine Myoglobin Reactivity Differences Involve Only T Cells and APC.* Because the irradiated spleen cells used as the source of APC are a heterogeneous population comprising several cell types, the A20 (I-A<sup>d</sup>) B cell lymphoma line was tested to see if the difference between native sperm whale and equine myoglobins was maintained. The results (not shown) indicate that when this line was used as an APC, the difference in dose-response curves between native sperm whale and equine myoglobins was maintained despite similar responses to the sperm whale and equine peptides. Therefore, the differences are not due to regulatory cells contaminating the APC source.

*Differences Between Native Equine Myoglobin and Peptide Can Be Overcome by Artificial Processing.* To determine the structural requirements for the reactivity differences of equine peptide versus equine native myoglobin, the response of clone 9.27 to apo-equine myoglobin and the largest CNBr fragment (56-131) that contains the T cell site was tested (Fig. 7). The responsiveness to apo-equine myoglobin was no greater than that to native equine myoglobin. Thus, the release of the heme group with some disruption of tertiary structure did not increase reactivity. However, the 56-131 CNBr fragment showed increased potency compared with the dose-response curve for equine myoglobin. The results suggest that the reactivity differences between equine peptide and native equine myoglobin may be due to lack of processing of the native molecules to a fragment corresponding to the minimal site and to the presence of structures on the naturally processed equine myoglobin that interfere with binding to I-A<sup>d</sup> or to the TCR of clone 9.27.

*Sperm Whale/Equine Myoglobin Reactivity Differences Are Maintained in the Same F<sub>1</sub> APC.* To determine whether the differences between sperm whale and equine myoglobin, and between the equine peptide and native molecule, are due to processing

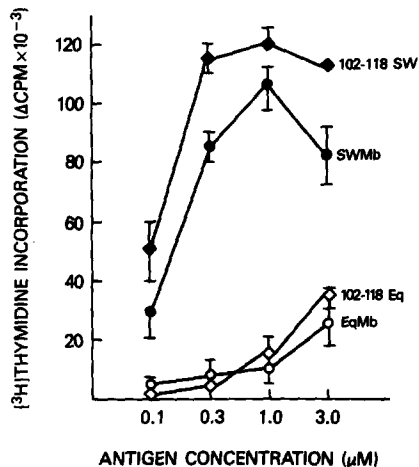


FIGURE 9. Proliferative response of sperm whale myoglobin-immune B10.D2 lymph node cells to whole myoglobin or the 102-118 peptides. Lymph node cells ( $3.5 \times 10^5$ ) were cultured with various concentrations of antigen. Control cultures without antigen gave 8798 (1.6) cpm. PPD responses were 150,653 (1.05) cpm for sperm whale-immune cells. Results are shown as  $\Delta$  geometric mean cpm of triplicate cultures ( $\times/\div$  geometric SEM).

differences in APC of different H-2 haplotypes, the ability of APC from (H-2<sup>d</sup> × H-2<sup>s</sup>)F<sub>1</sub> mice to present both proteins to clone 9.27 was tested. The H-2<sup>s</sup> haplotype was chosen because experiments above indicate that this strain is able to process and present the 102-118 region of equine myoglobin efficiently. The reactivity difference between sperm whale and equine myoglobin and peptides was maintained in the F<sub>1</sub> APC (Fig. 8). No response was observed on H-2<sup>s</sup> APC either alone or in the presence of any of the antigenic stimuli. These results suggest that differences in the reactivities of sperm whale and equine myoglobin were not simply due to MHC-linked processing or the availability of all antigenic fragments but were dependent on the I-A molecule used in presentation.

*Response of Sperm Whale Myoglobin-immune B10.D2 Lymph Node Cells to the 102-118 Region Indicates that Clone 9.27 Is Unusual But Not Unique.* Further investigations were carried out to determine whether the differential ability of clone 9.27 to recognize whole sperm whale versus equine myoglobin compared with the peptides was related to unique features of antigen recognition by this clone or reflected a generalized inefficiency of processing this site in equine myoglobin. B10.D2 mice were immunized with equine or sperm whale myoglobin, and lymph node proliferative responses to whole myoglobin and 102-118 peptides were assessed (Fig. 9). Results from sperm whale-immune lymph node cells confirmed previous studies that the response in this strain is predominantly directed to the 102-118 region, because this peptide was as stimulatory as sperm whale myoglobin. In several experiments, there was only a small response to equine myoglobin and a small but variable response to 102-118eq. Thus, it appears that 9.27 is probably a less common but not unique clone in being fully crossreactive at the peptide level.

*Comparison of Native Versus Peptide Response for Other Epitopes.* Results in the previous sections indicate that responsiveness to a particular determinant may depend on the structural environment of the determinant, as well as any intrinsic properties of the site involved in Ia binding. We therefore extended the above studies by investigating the crossreactivity with the native molecule after immunization with the different peptides. Immunization of B10.D2 mice with native sperm whale myoglobin elicits

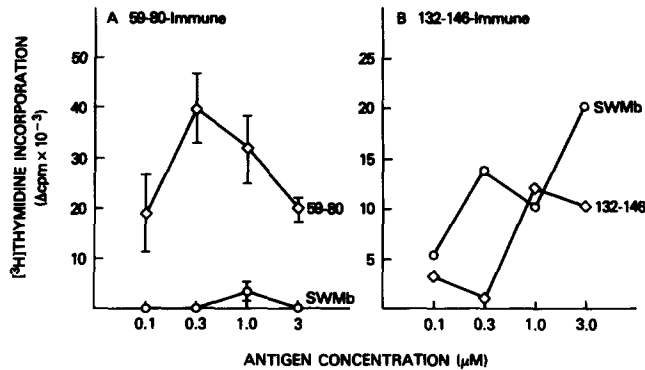


FIGURE 10. Proliferative response of peptide-immune lymph node cells from B10.D2 ( $H-2^d$ ) mice to whole sperm whale myoglobin and peptides. (A) mice immunized with 4 nmol of peptide 59-80; (B) mice immunized with 4 nmol of 132-146. Culture conditions and expression of results as in Fig. 4. Background cultures gave 11,665 cpm for B10.D2 cells immune to 59-80 and 5320 cpm for B10.D2 cells immune to peptide 132-146. PPD responses were 68,517 and 57,484 cpm in A and B, respectively.

a response predominantly to the 102-118 region, with only a small response to 59-80 and 132-146 (27-29). However, after immunization with the peptide 59-80, B10.D2 lymph node cells showed a good response to this peptide that was poorly recalled by whole myoglobin (Fig. 10 A). In contrast, after immunization with peptide 132-146, B10.D2 (Fig. 10 B) and B10.S (not shown) T cells responded moderately well to the whole protein as well as to the homologous peptide. The lymph node cells appeared to be specific for these peptides, because the peptides from other regions included as controls did not stimulate above background levels.

### Discussion

In this study we have provided two lines of evidence that antigen processing and the structural environment of a T cell determinant may influence the magnitude of the antigen-induced MHC-restricted proliferative response of T helper cells, the immunodominance of particular epitopes, and even the apparent fine specificity of isolated T cell clones. This may result from differences in processing between homologous proteins (such as equine or sperm whale myoglobin) or from structural differences between the fragments of a protein produced by natural processing and the synthetic peptides containing the core antigenic determinant. In the latter case, even if homologous proteins are processed equivalently, the resulting fragments may differ in hindering structures outside the core antigenic site. Such hindering structures in protein variants could account for the difference in response of clone 9.27 to native equine and sperm whale myoglobins, as well as for the heteroclicity of REL compared with HEL cited above (15, 30).

The first line of evidence was that B10.BR mice, which appear to be T cell non-responders to the 102-118 determinant after immunization with equine myoglobin, turned out to be good responders to this site in association with  $I-A^k$  after immunization with the synthetic 102-118 peptide. Thus, neither a gap in the T cell repertoire nor a failure of the epitope 102-118 itself to bind to  $A^k$  can account for the low responsiveness to this site when  $H-2^k$  mice are immunized with whole equine myoglobin. Also, since monoclonal populations show the same difference in response to whole myoglobin versus peptide, the difference cannot be due to suppressor T

cells specific for another epitope on the protein (30). Therefore, we looked for an alternative explanation. The difference in responsiveness to region 102-118 in B10.BR mice after immunization with whole myoglobin or peptide may be related to the requirement for processing of the native molecule. A comparison of the dose-response curves to whole myoglobin versus peptide for individual clones showed that, irrespective of fine specificity, the native protein was only 1.5 to 3 times less potent on a molar basis for B10.S clones compared with at least 10 times less potent for B10.BR clones. This difference was also apparent for 102-118-immune B10.S and B10.BR lymph node cells, because the B10.BR response, in contrast to the B10.S response, was not recalled well by equine myoglobin *in vitro*. A similar observation recently has been described with HEL, where immunization with peptide T11(74-96) induces a strong proliferative response in B10.A mice, which is not recalled by HEL (30, 31). Cultures of T cells from T11-immune B10 mice, however, show excellent HEL-induced recall under similar conditions (30, 31).

In the myoglobin system, B10.S and B10.BR mice have the same B10 background genes and would therefore be expected to share identical non-MHC processing genes. Since F<sub>1</sub> APC presented whole equine myoglobin no better than B10.BR APC to B10.BR T cells, the observed differences were not simply due to the generalized availability of all antigenic fragments, as might be expected if an MHC-linked processing difference distinguished H-2<sup>k</sup> from H-2<sup>s</sup>. Rather, the difference depended on the MHC restriction of the responding T cell. There has been some speculation that Ia molecules themselves may guide processing by binding the immunogenic peptides and somehow protecting them from further enzymatic digestion (32). For this to be the explanation for the results in the present study, peptides would have to be handled differently by APC and follow a different intracellular pathway from that of whole proteins, perhaps avoiding lysosomal degradation enzymes. A more likely explanation, however, may be that native myoglobin is processed down to an entity larger than 102-118 and that flanking regions interfere with binding to I-A<sup>k</sup> but not to I-A<sup>s</sup>. The interference may be by steric hindrance or by an effect on peptide conformation. The minimum length of the determinant is slightly different for B10.S and B10.BR clones (102-115 for the former and 106-117 for the latter) (manuscript in preparation). Thus, hindering structures in the flanking regions surrounding the 102-118 region in the naturally processed myoglobin product may play a major role in reducing the affinity of binding to I-A<sup>k</sup> but not to I-A<sup>s</sup>, and therefore, such hindering structures are MHC restricted.

Studies with the 102-118sw-specific I-A<sup>d</sup>-restricted clone 9.27 provided the second independent line of evidence that structural differences between equine and sperm whale myoglobin that are outside the epitope itself and distinct from those minimally necessary for recognition of peptide/Ia may contribute to the differences in responses. These differences may be related to the way murine APCs process equine versus sperm whale myoglobin, but the F<sub>1</sub> APC studies excluded actual MHC-linked differences in processing. Alternatively, the two proteins may be processed equivalently, but the natural product of processing equine myoglobin may contain hindering structures that interfere with binding specifically to A<sup>d</sup> but not A<sup>s</sup> or to the receptors of specific T cells. Such hindering structures could also explain the heteroclitic response to REL compared with HEL by C57BL/6 mice (15, 30). However,

in the latter case, sequence differences that influenced the response mapped outside the antigenic site, whereas in the case of clone 9.27, the only critical difference between sperm whale and equine myoglobin that could be mapped with a panel of 15 sequence variants mapped to residue 109, within the minimal determinant 106–118 defined with peptides (27, 28). Nevertheless, at the peptide level, this clone did not appear to discriminate between the sperm whale and equine sequences. This makes it unlikely that the difference in responsiveness to sperm whale versus equine myoglobin for this particular clone is related either to the fine specificity of the TCR for the core antigenic site or to different binding affinities of the core antigenic site for I-A<sup>d</sup>, as is the case for cytochrome *c* (33). Because none of the H-2<sup>d</sup> T cells specific for 102–118 responded well to native equine myoglobin, regardless of their ability to discriminate Asp versus Glu at position 109 in the peptide, it is likely that the postulated hindering structure interferes with binding to A<sup>d</sup> rather than to the specific receptor of clone 9.27. Alternatively, the hindering structure may bind to the epitope itself, analogous to the distant residue of cytochrome *c* that has been suggested to bind Lys 99 (34). If this structure binds better to Asp 109 than to Glu 109, it could explain the mapping of the equine/sperm whale difference to residue 109.

Further immunizations with other peptides revealed some new reactivities that had not appeared after priming with whole myoglobin. Most interesting was the response to the 132–146 region of sperm whale myoglobin, which was ~10% of the response to 102–118 in myoglobin-immune lymph node cultures (28, 35). Direct binding studies have, however, demonstrated that 132–146 binds to its restriction molecule (I-E<sup>d</sup>) with as relatively high affinity as 102–118 binds to I-A<sup>d</sup> (6). Immunization with this peptide induced a good response to the peptide that was recalled well by whole myoglobin *in vitro*. Therefore, in contrast to the observations described above for the 102–118 site, there was apparently no difficulty in processing myoglobin to produce a natural fragment that behaved similarly to the synthetic one. Previous studies have demonstrated that the T cell response to sperm whale myoglobin is primarily directed to 132–145 if APC that contain only I-E<sup>d</sup> were used to stimulate polyclonal T cells, but not if both I-A<sup>d</sup> and I-E<sup>d</sup> are present (28). If the two determinants are on the same processed fragment, internal competition between the I-A and I-E molecules for the same cleavage fragment may result, as also suggested by Gammon et al. (30). In contrast, immunization with 59–80 did not elicit T cells that crossreacted with the native molecule. This fragment of sperm whale myoglobin behaved in H-2<sup>d</sup> mice more like the equine 102–118 peptide in H-2<sup>k</sup> mice described above.

These constraints on crossreactivity between native proteins and the corresponding peptide epitopes, in cases where neither a gap in the T cell repertoire nor a failure of the synthetic epitope to bind to the appropriate MHC molecule exists, indicate that antigen processing may be another major mechanism determining *Ir* gene effects and immunodominance. Taken together, the results can best be explained by the existence of MHC-specific hindering structures on the products of natural processing. These structures and constraints will also be important in the development of synthetic peptide vaccines to induce T cell immunity, because such vaccines must elicit T cells that can crossreact with the native protein antigen as, for example, in recent studies of hepatitis (36), malaria (37), and AIDS (38).

### Summary

Two lines of evidence in the current study indicate that antigen processing is a major factor, in addition to MHC binding and T cell repertoire, that determines *Ir* gene responsiveness and epitope immunodominance. First, immunization with synthetic peptides of myoglobin sequences revealed new reactivities that had not appeared after priming with native myoglobin. For example, B10.S mice (H-2<sup>s</sup>) immune to equine myoglobin predominantly responded to peptide 102–118, whereas there was little, if any, response to this peptide in B10.BR (H-2<sup>k</sup>) mice immunized with native equine myoglobin. However, after immunization with the 102–118 peptide, both strains responded to the peptide. After *in vitro* restimulation, B10.BR T cells responded as well as B10.S T cells. Similarly, some individual 102–118-specific T cell clones from mice of both haplotypes showed similar dose responses and fine specificity patterns. Thus, low responsiveness to this site is due neither to a hole in the repertoire nor to a failure to bind to the appropriate MHC molecule. An alternative explanation was suggested by the observation that, whereas B10.S T cells from peptide 102–118-immune mice responded almost as well to whole myoglobin as to the peptide, the B10.BR T cells from peptide immune mice, while responding well to peptide, were poorly stimulated by whole myoglobin. Thus, the product of natural processing of equine myoglobin probably has hindering structures in the regions flanking the core epitope 102–118 that interfere with presentation by I-A<sup>k</sup> but not I-A<sup>s</sup>.

The second line of evidence that processing of native myoglobin may influence the apparent specificity of the T cell response was obtained using the I-A<sup>d</sup>-restricted sperm whale myoglobin 102–118-specific clone 9.27. This clone discriminated readily between whole sperm whale myoglobin and equine myoglobin, but it did not distinguish between peptides corresponding to 102–118 of the sperm whale and equine sequences. This distinction between equine peptide and native equine myoglobin could be overcome by artificial “processing” of equine myoglobin with cyanogen bromide. In both sets of experiments, F<sub>1</sub> APCs that present the same epitope well to T cells of another haplotype failed to overcome the defect, which was therefore not due to the availability of different processed cleavage fragments in APC of different haplotypes, as would be expected if there were MHC-linked processing. Thus, the differential responses to peptides versus native molecule for both I-A<sup>d</sup>- and I-A<sup>k</sup>-restricted clones appeared to depend on the restricting molecule used. The study was extended to immunization of H-2<sup>d</sup> mice with peptides 59–80 and 132–146 of sperm whale myoglobin, which also revealed new reactivities that were not a major part of the response when priming with the native molecule, even though both peptides bind with high affinity to I-A<sup>d</sup> (39) and I-E<sup>d</sup> (6), respectively. The 132–146-immune T cells from B10.D2 and B10.S mice, in contrast to 102–118-immune T cells from B10.BR mice, responded well to native myoglobin. Taken together, the results suggest that the fragments produced by natural processing may bind Ia molecules differently from the corresponding synthetic peptides. The existence of MHC-restricted hindering structures after natural processing may have important implications for the development of synthetic peptide vaccines that, although immunogenic themselves, may not necessarily elicit a response that crossreacts with the native molecule.

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