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Duration of Alloantigen Presentation and Avidity of T Cell Antigen Recognition Correlate with Immunodominance of CTL Response to Minor Histocompatibility Antigens

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CD8 T lymphocytes (CTL) responsive to immunodominant minor histocompatibility (minor H) Ags are thought to play a disproportionate role in allograft rejection in MHC-identical solid and bone marrow transplant settings. Although many studies have addressed the mechanisms underlying immunodominance in models of infectious diseases, cancer immunotherapy, and allograft immunity, key issues regarding the molecular basis of immunodominance remain poorly understood. In this study, we exploit the minor H Ag system to understand the relationship of the various biochemical parameters of Ag presentation and recognition to immunodominance. We show that the duration of individual minor H Ag presentation and the avidity of T cell Ag recognition influence the magnitude and, hence, the immunodominance of the CTL response to minor H Ags. These properties of CTL Ag presentation and recognition that contribute to immunodominance have implications not only for tissue transplantation, but also for autoimmunity and tumor vaccine design.


B one marrow transplantation is a major therapeutic modality for the treatment of blood cell disorders, including autoimmune diseases. Despite donor-recipient MHC class I and II alloantigen compatibilities, fatal graft-vs-host (GVH) disease can ensue due to CD8 T lymphocyte (CTL) responses triggered by non-MHC-encoded minor histocompatibility (minor H) Ags. Minor H Ags are self peptides derived from proteolytic processing of normal cellular proteins; they are presented to T cells by MHC class I and class II molecules (1–4). Immune responses to minor H Ags result either because the recipient carries a null allele (5, 6) or due to differential or induced expression (7), differential processing (8), or amino acid sequence variation within the peptide epitope (9–12). Thus, variant peptides, ranging from completely foreign to those with subtle differences, when presented by the donor class I molecule, are sufficiently alloantigenic to contribute to the GVH response.

C57BL/6 mice share H2b with BALB.B and 129 mice, but differ at numerous minor H loci (13). Nevertheless, immunization of C57BL/6 mice with BALB.B or 129 minor H Ags results in CTL responses that are primarily directed against H2Kb-restricted H60, H4b, and H28, as well as H2Db-restricted H7b, H13b, and HY alloantigens. Interestingly, the magnitude of the response to H60 and H4b is greater than that toward the other minor H Ags (10, 14–16). The mechanism(s) underlying this hierarchic T cell response, termed immunodominance, is poorly understood.

CTL responses to viral and bacterial Ags indicate that multiple aspects of Ag processing, presentation, and recognition impact immunodominance of class I-restricted Ags (reviewed in Refs. 17 and 18). Epitope density on APCs could be a critical factor in determining immunodominance because it influences the duration (dwell time) of CTL-APC contact and, hence, the strength of the T cell signal (19). Epitope density per cell reflects the sum of processing efficiency, TAP transport, the affinity of the peptide (p)/MHC class I interaction, and the rate of class I denaturation/deggregation at the cell surface. Dominant epitopes are recognized by CTL with high functional and structural avidity between the p/MHC complex and the TCR (20, 21). Moreover, viral and bacterial proteins are, in general, expressed in high quantities upon infection (17). Several pathogens also shut off host protein synthesis and, hence, derived epitopes have very little competition for MHC class I binding (22). Like autoantigens and tumor Ags, minor H Ags are derived from normal cellular proteins, and, hence, they are likely to differ from viral and bacterial proteins in their kinetics and levels of expression. Furthermore, epitopes derived from pathogens differ extensively from self (23–26), whereas many minor H Ag-derived epitopes differ only subtly from self (9–12). Therefore, the differences between viral Ags and alloantigens require that the principles of immunodominance to minor H Ags be empirically determined.

CTL biology can also contribute to immunodominance. Importantly, most studies have suggested a high precursor CTL (pCTL) frequency against dominant Ags (15, 27–29). This latter property is especially true of immunodominant H60-specific pCTL frequency (14–16), which is unusually high, even higher than those against dominant viral epitopes (14). Because of this unusually high pCTL frequency, responses to H60 can be elicited in mixed leukocyte culture (MLC) or by stimulating naive responders with epitope-pulsed syngeneic spleen cells (14). T cell competition (30,
31) could partly underlie immunodominance of minor H Ags, yet we found very little evidence for this mechanism to explain the immunodominance of H60 over minor H Ags (14).

To define the molecular mechanism(s) that influences immunodominance of minor H Ags, factors governing their presentation and recognition were analyzed in detail. We found that the duration of Ag presentation and the strength of Ag recognition correlated best with immunodominance. This finding has important implications not only for tissue transplantation, but also for a more general understanding of key mechanisms responsible for immunodominance.

Materials and Methods

Animals

C57BL/6j, 129/SvJ, C.B10-H2aLilMcdJ (BALB.B), C57BL/6.C-H60/H2cr (H60 congenic), B10.CE-H13Aa/(30N)x/Sn (H13 congenic), and B10.129-H40aH67/Sn (H4 congenic) mice (The Jackson Laboratory, Bar Harbor, ME) were bred and maintained according to Vanderbilt University’s Institutional Animal Care and Use Committee policies.

Cell lines

RMA-S, T2Kb, and T2Db (gifts from K. Karre, University of Stockholm, Stockholm, Sweden, and P. Cresswell, Yale University, New Haven, CT) cells were maintained, as described (10).

Peptide synthesis

Synthetic peptides (Table I) were synthesized by Fmoc chemistry (Pennsylvania State University College of Medicine, Hershey, PA) (10, 32, 33). Peptides and proteins were directly isolated and fractionated by reversed phase (RP) chromatography (C18 DEvelosil, 25 mm, 120 Å, 5 mm; Alltech Associates, Deerfield, IL) as described (32). Peptide elution was monitored at 214 and 280 nm, and two-drop fractions were collected. Target reconstitution. Peptide fractions were collected.

Isolation and characterization of naturally processed MHC class I-associated peptides

Peptide isolation and fractionation. Peptides and proteins were directly precipitated from splenocytes with 0.1% v/v trifluoroacetic acid (10, 34). Peptides in the soluble fraction were separated from peptides by Centricon 3 filtration, and fractionated by reverse phase (RP) chromatography (C18 250 × 1 mm, 120 Å, 5 mm; Alltech Associates, Deerfield, IL) as described (32). Peptide elution was monitored at 214 and 280 nm, and two-drop fractions were collected.

Target reconstitution. Peptide fractions were diluted in water, and trifluoroacetic acid and acetonitrile were evaporated under vacuum. Approximately 20 μl of each concentrated peptide fraction was resuspended in 100 μl of RPMI 1640 supplemented with 5% FCS and used in CTL assays.

Generation of minor H Ag-specific CTL, CTL clones, and 51Cr release assay

Minor H Ag-specific CTL and CTL clones were generated and maintained, as described (35–37). 51Cr release assay was performed according to standard protocols; E:T ratios are indicated in the figure legends. Target cell reconstitution assay was performed, as described by us (10), and the data are represented as percent specific lysis.

Peptide-binding assay

Rescue of class I expression in RMA-S cells by allele-specific peptides was determined, as described (10). H2Kb and H2Db were detected using biotinylated 28-8-6, after staining with streptavidin-PE (BD PharMingen, San Diego, CA), as described (10). Flow cytometry was performed using FACSCalibur (BD Biosciences, San Diego, CA) and analyzed using CellQuest version 3.0 (BD Biosciences).

Relative affinity (Kd) of class I peptide binding was calculated from specific mean fluorescence intensity (MFI); difference between total MFI at a defined peptide concentration and background MFI derived from ligand-free class I expression to the same RMA-S cells using nonlinear regression analysis fitted to classical Michaelis-Menten kinetics (Prism 3.02; GraphPad, San Diego, CA). Michaelis-Menten equation was used to calculate the Kd because Scatchard and Lineaweaver-Burk transformations, which use linear regression analyses, amplify any variation of the data from the linear curve (discussed below in detail).

MHC class I peptide dissociation assay

RMA-S cells (3.0–5.0 × 106) maintained at 26°C for 18–24 h were incubated with 1 μM peptide in duplicate for 45 min at 26°C. Peptide-pulsed cells were washed to remove unbound peptide and chased at 37°C for the indicated time intervals; cells were washed to remove the released peptides, and class I remaining at the cell surface was detected with biotinylated 28-8-6. Data are presented as percentage of maximum MFI, which was fitted to classical Michaelis-Menten kinetics from which td/2 of class I peptide interaction was calculated.

Cell surface Ag t1/2 determination

T2Kb or T2Db cells were pulsed with synthetic minor H peptides, washed extensively, and chased for the indicated times in presence of 5 μg/ml brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO). Two hours before the end of chase, they were 51Cr labeled and used as targets in a standard Cr release assay at an E:T of 10. Splenocytes from 129/SvJ mice free of RBC were resuspended at 2 × 106 cells/ml and stimulated with 10 μg/ml LPS and 2 μg/ml Con A. After 45–48 h, splenocyte blasts were washed and resuspended in medium, treated with BFA, and used in a standard 51Cr release assay, as described above. Low concentration of BFA (1 μg/ml) was present during 51Cr labeling and through the CTL assay because the effect of BFA is reversible upon withdrawal.

Intracellular cytokine staining (ICS)

Freshly harvested responders or those from MLC were stimulated with the indicated doses of peptide-pulsed T2Kb or T2Db for 5 h at 37°C in medium supplemented with 5 μg/ml BFA. Cells were then surface stained with anti-CD8a Ab, washed, and then permeabilized with Cytofix/Cytoperm kit (BD Pharmingen), according to the manufacturer’s protocol. Staining for intracellular IFN-γ was performed using XMG1-2-PE Ab (BD Pharmingen) and analyzed by flow cytometry. For normalizing percentage of CD8 IFN-γ response, background CD8 IFN-γ staining obtained using a control peptide was subtracted from the specific minor H Ag-elicted response. Data are normalized by determining the ratio of specific response at a given peptide concentration to maximum response, which is usually the response obtained from the highest concentration of the stimulating peptide.

Preparation and use of class I-peptide tetramers

Preparation and specificity of pK2b and pD2b tetramers have been described (10, 15). Freshly isolated responder splenocytes were incubated with indicated tetramer-PE and anti-CD8a FITC for 2 h at 37°C. After extensive washing, cells were fixed and analyzed by flow cytometry.

Determination of relative avidity

To determine the relative avidity (Kd) between the tetrameric Ag and its cognate TCR, isothermic binding assay of p/class I tetramers was performed. Indicated concentrations of tetramers were used to steady state

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Table I. The origin of MHC class I-restricted allelic variants of mH alloantigen

<table>
<thead>
<tr>
<th>H2/minor H</th>
<th>C57BL/6</th>
<th>BALB.B</th>
<th>129</th>
<th>Incompatibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kb/H60</td>
<td>nd</td>
<td>LTNYRNL</td>
<td>b</td>
<td>B6 is null strain</td>
</tr>
<tr>
<td>Kb/H4</td>
<td>a</td>
<td>SGVYIHL</td>
<td>b</td>
<td>Variation at P1, P3</td>
</tr>
<tr>
<td>Kb/H28</td>
<td>nd</td>
<td>ILENFPR</td>
<td>b</td>
<td>Induced</td>
</tr>
<tr>
<td>Db/H7</td>
<td>a</td>
<td>KAPDNRDLT</td>
<td>b</td>
<td>Variation at P7</td>
</tr>
<tr>
<td>Db/H3a</td>
<td>a</td>
<td>ASPCNSTVL</td>
<td>a</td>
<td>Variation at P1, P4</td>
</tr>
<tr>
<td>Db/H13</td>
<td>a</td>
<td>SSVGVVWYL</td>
<td>b</td>
<td>Variation at P4</td>
</tr>
<tr>
<td>Db/H6</td>
<td>nd</td>
<td>WMHHNMNTLI</td>
<td>nd</td>
<td>Male Ag</td>
</tr>
</tbody>
</table>

*nd, No described allele; bold, anchor residues; bold underline, amino acid variation between strains.
(>2 h) label TCR-bearing cells at 4°C in 100 μl of 2% FCS and 0.05% w/v Na3C03-supplemented PBS to prevent capping and internalization of the TCR. \( K_a \) was calculated from the specific MFI (difference of the total MFI at a defined tetramer concentration and background MFI derived from irrelevant tetramer binding to the same cells) using data fitted to classical Michaelis-Menten kinetics, as described above.

We have performed extensive Ag/TCR-binding studies in which the binding isotherms were transformed by Michaelis-Menten equation to determine the \( K_a \) of the said interactions (38). The results so obtained were compared with those obtained by Scatchard analysis. Results from both analyses agreed with each other (38). If linear regression analysis is used, as in Scatchard or Lineweaver-Burk transformation, deviation from the linear plot will be highly amplified and would bias the data. Deviation from a linear curve is of particular concern when binding studies are performed using live cells. Therefore, the use of Michaelis-Menten kinetics, which uses nonlinear regression analysis of the binding data, was preferred over the other methods.

**Results**

**Competition for access to Ag is not a factor that influences immunodominance of H60 and H4b alloantigens**

To determine the conditions required to elicit CTL responses to minor H Ags, C57BL/6 mice were immunized with either BALB.B or individual minor H locus congenic male splenocytes. Alloantigen-specific CTL response was analyzed using tetramers either directly ex vivo or following in vitro stimulation in MLC. Robust H60- and H4b-specific CTL responses were detectable with specific tetramers in ex vivo analysis after immunization with BALB.B splenocytes (Fig. 1A). In striking contrast, neither H13b (Fig. 1A)- nor HY (data not shown)-specific responses were detectable with specific tetramers under similar conditions of immunization and analysis. However, after in vitro stimulation with irradiated BALB.B splenocytes, tetramer-positive H13b (Fig. 1A)-, but not HY (data not shown)-specific CTL were detectable. The H60-specific response analyzed both ex vivo and after MLC was always greater than that against H4b, which was 15- to 20-fold greater than the response to H13b (Fig. 1A). Thus, under conditions in which all minor H Ags tested are potentially expressed by the splenocytes used for immunization, the CTL response hierarchy is H60>H4b>H13b>HY.

When the immunizing splenocytes express multiple minor H Ags, the CTL response hierarchy observed could be due to competition for access to Ag. Immunization of C57BL/6 mice with splenocytes from mice congenic at a single minor H locus restricts the CTL response to the encoded minor H Ag. However, because immunization with single minor H Ag does not elicit CD4 T cell help, C57BL/6 mice were immunized with splenocytes from male congenic mice because the male chromosome encodes a strong H28, H7b, H3a b, and HY peptide. Two-fold dilutions of fractions containing each of the naturally processed minor H Ag-derived peptides were tested in a target cell reconstitution assay. Independent titrations of 100 fmol of the synthetic peptides fractionated and tested in parallel were used to generate a standard curve (Fig. 2) from which the copy number of the naturally processed peptides was estimated. Surprisingly, although immunodominant, the copy number of H60 and H4b peptides was 6- to 10-fold lower than H13b peptide (Table II). The copy number of H28, H7b, H3a b, and HY peptides was below the detection level.
Table II. Summary of biochemical and functional parameters of mH Ag presentation and recognition

<table>
<thead>
<tr>
<th>mH Ag</th>
<th>Densitya per Cell</th>
<th>Class I-Binding Parameters</th>
<th>Surface t1/2 (hours)</th>
<th>Functional Avridity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kd (nM)</td>
<td>t1/2 (hours)</td>
<td>Syntheticb</td>
</tr>
<tr>
<td>H60</td>
<td>~30–90</td>
<td>0.8 ± 0.05</td>
<td>4.8 ± 0.1</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>H28</td>
<td>Low</td>
<td>82.0 ± 2.9</td>
<td>1.1 ± 0.04</td>
<td>~2.0</td>
</tr>
<tr>
<td>H4b</td>
<td>~30–80</td>
<td>0.8 ± 0.04</td>
<td>1.8 ± 0.1</td>
<td>&gt;6.0</td>
</tr>
<tr>
<td>H13b</td>
<td>~390</td>
<td>3.8 ± 1.3</td>
<td>1.1 ± 0.37</td>
<td>~6.0</td>
</tr>
<tr>
<td>H7b</td>
<td>Low</td>
<td>7.0 ± 0.4</td>
<td>1.6 ± 0.18</td>
<td>ND</td>
</tr>
<tr>
<td>H3ab</td>
<td>Low</td>
<td>7.0 ± 0.8</td>
<td>1.7 ± 0.65</td>
<td>ND</td>
</tr>
<tr>
<td>HY</td>
<td>250.0 ± 4.6</td>
<td>1.3 ± 0.09</td>
<td>~2.0</td>
<td>~3.0</td>
</tr>
</tbody>
</table>

a Density indicates the number of epitopes generated per cell; it does not mean the number of Ags presented on the cell surface; based on Fig. 2 and data not shown (n = 2).
b See Fig. 3A (n = 3).
c See Fig. 3B (n = 3).
d Based on the t1/2 of 1.0 mM peptide-pulsed targets shown in Fig. 4A.
e See Fig. 4B (n = 2).
f See Figs. 5A, and data not shown (n = 2).
g See Fig. 5B (n = 3).

The lack of correlation between minor H peptide density and immunodominance could be because the immunodominant peptides bind their respective class I molecule with high affinity, while the recessive peptides bind with weak affinity. Therefore, we determined the concentrations of the peptides (see Table I) required to stabilize class I on the surface of TAP-deficient RMA-S cells. From the binding isotherms (Fig. 3a), the affinity of peptide/class I interaction was calculated (see Materials and Methods). The affinity (Kd) of both H60 and H4b (the naturally processed H60 peptide is a phosphopeptide (10)) for H2Kb was higher than H28 for H2Kb and H13b, H7b, H2a, and HY for H2Db (Table II). Furthermore, the class I epitope dissociation rates reflected the Kd of the minor H peptide/class I interactions (Fig. 3B; Table II).

The above biochemical binding parameters partly explain why H60 is immunodominant, but do not provide clues to the basis for H4b immunodominance. Therefore, the functional t1/2 of H60/Kb, H4b/Kb, and H28/Kb as well as H13b/Db and HY/Db complexes on the surface of target cells was determined. Functional t1/2 can be determined by two approaches: in the first, 1–1000 nM peptide-pulsed T2Kb or T2Db cells, depending on the epitope being tested, were chased after removal of excess peptides, in the presence of BFA to block the trafficking of newly synthesized p/MHC class I complexes from the endoplasmic reticulum to the cell surface. The choice of T2Kb and T2Db cells was based on our finding that RMA and RMA-S cells had high background in CTL assays (data not shown) possibly because BFA may have altered the physiology of these cells. Two hours before the end of chase, cells were labeled in the presence of BFA (because the effect of BFA is rapidly reversible upon withdrawal).

The data are represented as relative percent specific lysis based on 100% value attributed to specific lysis of targets at the beginning of the chase. Because the lowest concentration of the peptide used to pulse the targets was at least 10-fold greater than the half-maximal concentration needed to sensitize targets for recognition by the CTL clones (data not shown), the variation in percent specific lysis at different peptide concentration was < 10% (Fig. 4A, boxed inset, and data not shown). As expected from the biochemical data described in the preceding paragraphs, the t1/2 of H60 peptide presentation was longer that of H60 peptide, which was similar to H13b (Fig. 4A). The t1/2 of H28 and HY peptide
presentation was similar, but shorter than for H60, H4b, and H13b peptide presentation (Fig. 4A).

A second approach was used to confirm the above results because the $t_{1/2}$ of presentation of the different synthetic minor H peptides, although showing some differences, was not as striking as the hierarchy of the CTL response to various minor H Ags. The lack of a striking difference may be due to the fact that the above method used supraoptimal/nonphysiological epitope density to determine the $t_{1/2}$ of Ag presentation. Thus, Con A- and LPS-stimulated BALB.B or 129 splenocytes were treated with BFA for varying periods of time; 2 h before the end of BFA treatment, blasts were $^{31}$Cr labeled and used as targets for minor H Ag-specific CTL clones. The functional $t_{1/2}$ of H60/Kb, H4b/Kb, and H13b/Db, and HY/Db complexes were longer than that of H28/Kb, H13b/Db, and HY/Db (Fig. 4B). These data using cells presenting physiological levels of minor H p/MHC complexes at the cell surface were similar to those obtained from peptide-pulsed and chased targets (Fig. 4A). They are consistent with the biochemical binding parameters of p/MHC class I complexes (Table II). Thus, the duration of Ag presentation can influence immunodominance.

Functional avidity partially correlates with immunodominance

Functional avidity, measured as sensitivity to epitopes in a lytic or IFN-γ response assay, is a measure of quantitative Ag recognition, which could influence immunodominance. To measure functional avidity, C57BL/6 mice were immunized twice, 1 wk apart, either with BALB.B or 129 splenocytes. A week after the second immunization, immune splenocytes were restimulated in vitro in a 5-day MLC. The expanded responder CTL were used to determine the sensitivity with which CTL recognize target cells pulsed with $10^{-6}$ to $10^{3}$ nM of synthetic minor H peptides. Note that the background in the assay was established using the same concentrations of SV40 T Ag-derived H2Kb-restricted epitope IV (epi-IV) and H2Dd-restricted epitope II/III (epi-III) peptides (for brevity and clarity, only the highest concentration is shown). The rank order of sensitivity was H60$>$$>$H4b$>$$>$H28 and H13b$>$$>$ HY (Fig. 5A; Table II).

CTL activity measured by a $^{31}$Cr release assay reflects the function of the entire CD8 T cell population generated against a specific minor H Ag. Therefore, it is not surprising that the H60- and H4b-specific CTL, which are elicited in large numbers (Fig. 1), are more sensitive to their cognate Ags (Fig. 5A). To determine the sensitivity of Ag
recognitation at a single cell level, a portion of the CTL generated in the above MLC was rested for 6 days in the presence of low levels of IL-2. These CTL were restimulated with \(10^{-5}\) (H60, H28, H4\(^b\), and H4\(^*\)) or \(10^{-4}\) (H13\(^b\), H7\(^b\), H3a\(^b\), HY, and H13\(^a\)) to \(10^6\) pM peptide-pulsed T2K\(^a\) or T2D\(^b\) cells for 6 h; BFA was added during the last 4 h of stimulation. The allelic variants H4\(^b\) and H13\(^b\) were used as negative controls. Cells stained for CD8 and intracellular IFN-\(\gamma\) revealed sensitivity order of H60\(>\)H4\(^b\)\(>\)HY\(>\)H28\(>\)H13\(^b\)\(>\)H7\(^b\)\(>\)H3a\(^b\) (Fig. 5B; Table II). The sensitivity of HY-specific CTL may have fallen out of the rank order in the ICS assay because in vitro restimulation in MLc may have set a bias. This argument is further supported by the fact that the ex vivo analysis of minor H Ag-specific CTL responses by ICS for IFN-\(\gamma\) revealed only H60-, H4\(^b\)-, and H28-specific activity and none against H13\(^b\), H7\(^b\), H3a\(^b\), or HY (data not shown). Despite differences in sensitivity, CTL to both the immunodominant and recessive alloantigens show high levels of IFN-\(\gamma\) as judged by the MFI (Fig. 5B insets). Taken together, the data suggest that the sensitivity of CTL against specific minor H Ag only partially reflects the hierarchy of this response.

There appears to be an apparent dichotomy between the more sensitive H2K\(^a\)-restricted and the less sensitive H2D\(^b\)-restricted Ag recognition in both lytic and ICS assays. This dichotomy may reflect diverging stimulatory capacities of T2K\(^a\) and T2D\(^b\) lines, which were used for minor H peptide presentation to specific CTL. Our finding that H2K\(^a\)-restricted H60- and H4\(^b\)-specific CTL are more abundant than H2D\(^b\)-restricted H13\(^b\)- and HY-specific CTL (Fig. 1) in C57BL/6 anti-BALB.B response suggests that the differential stimulatory capacities of T2K\(^a\) and T2D\(^b\) are less likely to have influenced the outcome of the functional avidity determinations described in this work.

**Structural avidity correlates with immunodominance**

Tetramers of p/MHC complexes permit measurement of relative structural avidity (\(K_{av}\)) of p/MHC-TCR interactions on live T cells. Thus, ex vivo and in vitro MLC-stimulated minor H Ag-immune C57BL/6 splenocytes, generated as described in Fig. 1, were incubated with increasing concentrations of p/MHC class I tetramers. From the binding isotherms (Fig. 6A), the avidity (\(K_{av}\)) of minor H p/MHC-TCR interaction was calculated, as described in...
Materials and Methods. Ex vivo binding studies revealed that the $K_{av}$ of H60/Ka for its TCR is ~2-fold higher than those of $^3$H4b/Ka and its TCR (Fig. 6A, top panel; Table III). However, ex vivo analysis of the $t_{1/2}$ of H60/Ka-TCR and $^3$H4b/Ka-TCR interactions revealed that the dwell time of these complexes on their respective receptors was similar (Fig. 6B, top panel; Table III). Therefore, H60 and H4b immunodominance could be the result of $K_{av}$ and optimal dwell time of minor H p/MHC-TCR interactions.

CTL responses against H28, H13b, and HY were barely detectable ex vivo (Fig. 1A), and, hence, the $K_{av}$ of these alloantigens for cognate TCR could not be measured. To empirically determine the $K_{av}$ and $t_{1/2}$ of H13b/D3b-TCR and HY/D3b-TCR interactions, splenocytes from mice immunized with either multiple BALB.B-derived (see Fig. 1A) or individual minor H Ags (see Fig. 1B) were restimulated once or twice in vitro (see Fig. 1, A and B). The resulting CD8+ and tetramer-positive cells were used to generate the Ag-binding isotherm (Fig. 6A, bottom panel) from which $K_{av}$ for each minor H p/MHC-TCR interaction was calculated. The $K_{av}$ of H13b/D3b-TCR and HY/D3b-TCR were similar, which was 2- to 4-fold lower than H60/Ka-TCR and $^3$H4b/Ka-TCR interactions (Table III). These data should be viewed with caution because in vitro stimulation may have selected for CTL, whose TCR may have high avidity. These and data not shown (see Materials and Methods).

Table III. Binding parameters of mH Ag/TCR interaction

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Analysisa</th>
<th>$K_{av}$ (nM)b</th>
<th>$t_{1/2}$ (minutes)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Exvivo-1</td>
<td>16.6</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>-2</td>
<td>14.8</td>
<td>7.2</td>
</tr>
<tr>
<td>Anti-BALB.B</td>
<td>First MLC-1</td>
<td>14.4</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>-2</td>
<td>17.6</td>
<td>7.4</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Exvivo-1</td>
<td>11.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Anti-congenic</td>
<td>First MLC-1</td>
<td>16.2</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>-2</td>
<td>18.1</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td>Second MLC-1</td>
<td>ND</td>
<td>ND</td>
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aSee Fig. 1 legend; -1 and –2 represent Expts. 1 and 2, respectively.
bCalculated based on the binding isotherms shown in Fig. 6A and data not shown (see Materials and Methods).

cCalculated based on tetramer dissociation curves shown in Fig. 6B and data not shown (see Materials and Methods).

ND, not determined because specific CTL was not generated (see Fig. 1B); in the case of H60 and H4b, second MLC was not performed because the first MLC significantly amplified the responding CTL population. The $K_{av}$ of H2Kb/H28/TCR interaction was determined to be >200 nM, which was calculated from the binding isotherm using specific tetramer and the H28-specific CTL clone, SPH28 (see Ref. 38).

Discussion

Minor H Ags are alloantigenic self peptides that derive from normal cellular proteins. Our goal was to take advantage of the now rich resources available for minor H Ag studies to systematically and comprehensively investigate the biochemical mechanism(s) underlying immunodominance to such endogenous Ags. Our data indicate that, among the constellation of factors that could contribute to immunodominance of minor H Ags, the duration of Ag presentation and the strength of alloantigen-TCR interaction correlate best with immunodominance.

Several models have been studied to elucidate the mechanism(s) underlying immunodominance to CTL Ags; the large majority of which focus on quantitative aspects of Ag presentation (reviewed in Refs. 17 and 18). The data from these studies indicate a lack of correlation between naturally processed epitope density and immunodominance (39–43). Previous studies, which have evaluated naturally processed minor H epitope density, have relied on peptides isolated from tumor cell lines (5, 7, 9, 12). To more closely reflect the minor H epitope density encountered in a transplant setting, we used freshly isolated splenocytes as the source of naturally processed peptides. Our data support the conclusion that epitope density does not necessarily correlate with immunodominance, in that immunodominant H60 and H4b peptides were less abundant than the recessive H13b peptide. However, given the heterogeneity of spleen cells, we cannot exclude the possibility that certain peptides are generated and presented in higher densities in professional APC, such as donor dendritic cells used for immunization.

From the data presented in this study, it is difficult to predict immunodominance of an Ag by determining epitope density and its MHC affinity. However, the duration of cell surface display of naturally processed CTL Ags did correlate with immunodominance. Consistent with our observation, immunodominance in the SV40-specific CTL response correlated with the duration of Ag presentation as well. Thus, H2Kb-restricted, SV40 epi-IV display extends over a longer period compared with recessive H2Db-restricted epi-I, epi-II/III, and epi-V (44). The influence of duration of Ag presentation in eliciting CTL response is also observed in experimental systems in which either dendritic cells pulsed with increasing quantities of epitopes (21) or cells genetically engineered to express increasing quantities of the peptide (45) are used as immunogens. Previous attempts at determining the duration of Ag presentation used synthetic peptide-pulsed target cells (46–48). Whereas such analysis has the potential to yield useful information, the differences observed in the present study were subtle and, hence, difficult to interpret. However, differences in functional $t_{1/2}$ are evident when the $t_{1/2}$ of naturally processed p/MHC complexes is analyzed, as reported in this work. The difference between the two assays may be

6672 CAUSES OF IMMUNODOMINANT CTL RESPONSES
due to the use of supraphysiological quantities of synthetic peptides in experiments attempting to determine the duration of Ag presentation. Therefore, the duration of presentation of naturally processed Ags at the cell surface should be empirically determined to obtain meaningful information on the functional $t_{1/2}$ of p/MHC complex.

The $t_{1/2}$ of the p/MHC complex at the cell surface has important implications for both direct and indirect Ag presentation. It is generally assumed that all p/MHC complexes assembled in cells are expressed at the cell surface, but this may not be the case (see Ref. 49). Our data indicate that $\sim 30$–80 $H^4$ and $\sim 390 H^3$ epitopes are generated by 129 splenocytes. Therefore, $H^3/D^b$ complexes should be 5–13 times more efficient at eliciting a CTL response. However, $H^4$-specific response dominates that against $H^3$.

Among parameters that could cause $H^4$ immunodominance is the number of $H^4/K^b$ complexes displayed at the cell surface. Neither the epitope density nor the $K_d$ of epitope/class I interactions provides a direct estimate of the number of p/MHC complexes displayed at the cell surface. However, the $t_{1/2}$ of p/MHC complexes as quantified in this study is a reflection of the number of Ags presented at the cell surface. Therefore, despite the generation of $\sim 390 H^3$ epitopes per cell, we predict that only a few $H^3/D^b$ molecules arrive at the cell surface, perhaps because they rapidly dissociate intracellularly compared with $H^60$ and $H^4$ p/K complexes (implied from the $K_d$ of p/class I interactions). Alternatively, all $\sim 390 H^3/D^b$ complexes arrive at the cell surface, but a large majority rapidly dissociate or turn over, and, hence, only a few molecules are left at the cell surface. Thus, the duration of Ag presentation at the cell surface appears to be a more relevant measure of immunogenicity, the rationale for which is discussed below.

Current evidence obtained from studies using T cell lines or clones and artificial APCs in vitro suggests that a T cell stops and begins signaling as the TCR recognizes one or a few molecules of the Ag. The sustenance of signaling requires recruitment of additional Ag molecules, and sustained signaling is essential for full T cell activation. Once activated, T cell-APC interface forms an immunological synapse. The maintenance of this synapse for several hours (>10 h) is essential for the elicitation of an effector function from the T cell. The stability of the synapse depends on the recruitment of more Ag-TCR complexes at the synaptic interface. The activation and the elicitation of effector function from naive T cells in vivo perhaps follow similar rules (reviewed in Ref. 50).

The activation and the elicitation of effector function from naive T cells in vivo perhaps follow similar rules (reviewed in Ref. 50). Nevertheless, the number of Ag molecules required to stop and signal naive T cells and the length of time required for eliciting effector function from them may be greater than those described above. It is in this context that the duration of Ag presentation may play a critical role in determining immunodominance of a T cell Ag in vivo. For example, the immunodominant minor H Ags $H^60$ and $H^4$ are presented for longer time periods, and, hence, they would be predicted to sustain the immunological synapse for a longer time compared with the recessive $H^3$ and $H^4$ Ag, which are presented for a shorter time at the cell surface. Ags with short $t_{1/2}$ may lead to premature dissolution of the synapse when the Ag dissociates from the receptor; the dissolution of the synapse then disrupts sustained T cell triggering. When Ags are in limited supply, as is the case with cross-presented Ags (see below), the disrupted synapse may never form again because new p/MHC complexes are not formed and, hence, do not arrive at the cell surface. Thus, the duration of Ag presentation can profoundly impact immunodominance.

Of the different minor H Ags studied in this work, $H^60$ and $H^4$ are cross-presented to specific CTL in vivo (14, 51). Cross-presentation requires the uptake of minor H Ags by host APC. Such Ags, although expressed continuously by the splenocytes used for immunization, are likely to be in limited supply because the responder APC must retrieve the minor H Ag from exogenous cellular sources. Under such circumstances, differences in the duration of Ag presentation at the cell surface could profoundly influence Ag recognition and, hence, the T cell response.

The duration of Ag presentation can directly influence the qualitative and quantitative aspects of Ag recognition and T cell response. The dwell time of Ag-TCR interaction has recently emerged as a critical parameter for effective T cell activation (52). Therefore, the dwell time of minor H p/MHC complex on cognate TCR would be predictive of immunodominance. Surprisingly, immunodominance in the system studied in this work did not correlate with dwell time of p/MHC-TCR interaction. Nevertheless, immunodominance correlated well with the $K_{sec}$ of minor H p/MHC-TCR interaction. Our preliminary data revealed that this correlation between $K_{sec}$ and immunodominance also extended to the SV40 model. Thus, the immunodominant SV40 T Ag-derived epitope-iIV/K b complex has a higher $K_{sec}$ for its TCR compared with the recessive epitope-IV/D b-TCR interaction (Y.Y., T. Schell, S. S. Tevethia, and S.J., unpublished data). Furthermore, 2C TCR mutants selected for high affinity for 2C p/L b complex also revealed that Ag recognition and response were driven by the strength rather than the dwell time of the Ag-TCR interaction (53). Thus, the $K_{sec}$ of p/MHC-TCR interactions is an important determinant of an efficient T cell response.

In summary, among a cohort of minor H Ags, $H^60$ and $H^4$ show the most consistent biochemical and functional features, including high affinity p/MHC binding and extended $t_{1/2}$. They also showed the most efficient Ag-TCR interaction parameters measured by tetramer-binding $K_{sec}$ and $t_{1/2}$. This convergence may elevate their immunodominance potential and could therefore be useful as predictors of GVH disease and the graft vs leukemia effect. Our findings also clarify the key factors, which contribute to immunodominant ligands in general. The level of protein expression can influence p/MHC density (45, 54), which in turn has the potential to increase the duration of cell surface Ag display and, hence, the ability to induce CTL response. Increased duration of Ag display could thus maximize the CTL responses in vivo, resulting in immunodominance.

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References

CAUSES OF IMMUNODOMINANT CTL RESPONSES