Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8191–8195, November 1988
Immunology

Peptide-mediated modulation of T-cell allorecognition

(antigen presentation/HLA class II major histocompatibility complex/influenza hemagglutinin)

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Communicated by D. B. Amos, July 25, 1988 (received for review June 17, 1988)

ABSTRACT **Antigen-specific helper T cells recognize a complex of peptide antigen and class II major histocompatibility complex (MHC) gene products. Whether T cells recognize MHC class II alloantigen by a similar mechanism or the native conformation of MHC molecules themselves has yet to be determined. The demonstration that peptide antigens bind directly and specifically to class II molecules has allowed us to examine the influence of foreign peptide binding on T-cell recognition of allogeneic MHC molecules. We report here that an immunodominant, HLA-DR1-restricted peptide of influenza virus hemagglutinin (HA residues 306–320) is able to modulate the recognition of alloantigen by human DR1-specific T-cell clones. For some T-cell clones, but not all, the HA peptide inhibited allorecognition in a dose-dependent manner. However, in one instance, the proliferative response to alloantigen was enhanced in the presence of HA peptide. These results suggest that the specificities of T-cell responses to allogeneic MHC molecules are heterogeneous, which may be influenced by different peptides occupying the class II MHC combining site and by the diversity of antigen-specific receptors of T lymphocytes recognizing the same MHC/peptide complex.**

T cells recognize foreign antigens in association with autologous molecules of the major histocompatibility complex (MHC) (1–3). In contrast, alloreactive T cells, which mediate tissue allograft rejection, *appear* capable of recognizing MHC alloantigens directly. However, this is not necessarily supported by recent crystallographic studies on the three-dimensional structure of the human class I MHC molecule (4, 5), which suggest the presence of a peptide associated with the presumed antigen combining site of the molecule. Additional evidence against direct allorecognition of MHC molecules is found in the somewhat surprising observation that T-cell clones that co-recognize nominal antigen and self MHC proteins are also able to recognize allogeneic MHC antigens in the absence of foreign exogenous antigen (6). This is further supported by more direct experiments demonstrating that murine T cells can recognize an HLA class I peptide presented by accessory cells (7). Furthermore, it has been demonstrated that synthetic peptides based on the HLA-A2 sequence are able to inhibit human cytolytic T-cell clones specific for the A2 class I molecule (8, 9). Thus, similar to recognition of nominal antigens, recognition of alloantigens may also require a ternary complex consisting of T-cell receptor, an allogeneic molecule of the MHC, and either a foreign or endogenous peptide.

Extending the early competitive inhibition studies by Werdelin (10), others (11, 12) have recently demonstrated that peptide antigens bind specifically to murine class II molecules. Therefore, if alloreactive T cells as well as antigenic peptides bind to spatially related sites on the MHC molecule, then it should be possible to examine their relative proximity in competitive inhibition types of experiments. This was tested in a murine system where a small minority of alloreactive T cells was inhibitable by high concentrations of antigen (13). In contrast to these studies, we have used peptide binding to the MHC molecule as a probe of human alloreactive T-cell specificity using an immunodominant (14) peptide corresponding to amino acids 306–320 of H_3 influenza virus hemagglutinin ($HA_{306-320}$). Since T-cell recognition of $HA_{306-320}$ has previously been shown to be restricted by HLA-DR1 (15), it should therefore bind to DR1 class II molecules and perhaps be able to inhibit DR1-specific alloreactive T-lymphocyte clones (TLCs) that recognize a site within close proximity to the peptide combining site. Indeed, a majority (five of eight) of TLCs was blocked by the addition of $HA_{306-320}$, whereas two clones were unaffected by peptide binding. Importantly, proliferation by one TLC was dramatically potentiated by the addition of $HA_{306-320}$ in the apparent absence of heteroclitic recognition (16). Our results suggest that T-cell receptor may interact with distinct regions of the class II molecule and that differential displacement of endogenous peptides by $HA_{306-320}$ may occur. The combined effects of these two mechanisms may account for the extensive heterogeneity of T-cell responses to alloantigens (17).

MATERIALS AND METHODS

Peptides. Peptides were synthesized as described (18). The sequences of the ragweed (RA) (residues 51–65) and HA peptides used in the studies reported here are described below:

RA_{51-65} Glu-Val-Trp-Arg-Glu-Glu-Ala-Tyr-His-Leu-Ala-Asp-Ile-Lys-Asp
$HA_{306-320}$ Cys-Pro-Lys-Tyr-Val-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr-Gly

T-Cell Clones. TLCs were generated as described (19). Three series of alloreactive TLCs were derived: series 61 (DR2;DRw13 anti-DR1); series 62 (DR2;DRw14 anti-DR1;DR2); series 63 (DR2;DRw13 anti-DR1;DR2). Therefore, DR1-associated allodeterminants should have been primarily recognized since DQ and DP antigens were shared. Briefly, peripheral blood lymphocytes (PBLs) were isolated by density gradient centrifugation over Ficoll/Hypaque and primed in the presence of an optimized concentration of irradiated allogeneic PBLs. After 6 days the primed lymphoblasts were fractionated on a Ficoll/Hypaque gradient and cloned by limiting dilution in the presence of T-cell growth factor and a fresh alloantigenic challenge. TLCs were expanded by serial exposure to T-cell growth factor and allogeneic feeder cells and frozen at −180°C before being

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Abbreviations: HA, hemagglutinin; LCL, lymphoblastoid B-cell line; MHC, major histocompatibility complex; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; TLC, T-lymphocyte clone; APC, antigen-presenting cell.

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thawed and screened in proliferation assays for their specificities on panels of allogeneic PBLs or lymphoblastoid B-cell lines (LCLs).

HLA-DR1-restricted TLCs specific for the $HA_{306-320}$ peptide (TLC HA1.7) were generated and characterized as described (14, 15).

Characterization of Allogeneic Stimulator Panels. PBLs were HLA typed using standard protocols for NIH microcytotoxicity, as described (19). HLA-DP typing was also performed as described elsewhere (20, 21) using specifically primed, polyclonal T-cell lines. Homozygous stimulator LCLs and their respective abilities to restimulate the alloreactive TLCs were characterized as part of the Tenth International Histocompatibility Workshop (22).

Proliferation and Inhibition Assays. Stimulator cells (PBLs or LCLs) were irradiated [3000 or 10,000 rads (1 rad = 0.01 gray), respectively] and resuspended at the indicated concentrations in RPMI 1640 medium supplemented with 10% human plasma, 2 mM L-glutamine, 25 mM Hepes buffer, 50 μg of gentamicin per ml, 100 μg of streptomycin per ml, 100 international units of penicillin per ml, and 25 international units of sodium heparin per ml. If peptide was to be used, it was added at the indicated concentrations 2–24 hr prior to the addition of TLCs. Responder TLCs were plated at 1×10^4 cells per well in supplemented medium. Triplicate cultures (200 μl) were incubated 48 hr at 37°C in 5% CO_2/air and then pulsed overnight with 1.0 μCi of [^{3}H]thymidine (1 Ci = 37 GBq). Proliferation, as correlated with incorporation of radiolabel, was measured by liquid scintillation spectroscopy and expressed as the mean ± SEM. It should be noted that alloreactive T-cell clones were added under suboptimal stimulating conditions in which the LCL with bound peptide would be limiting at low cell concentrations. Suboptimal conditions ensured that potential inhibitory effects of the peptide could be detected.

Monoclonal Antibody (mAb) Blocking Assays. mAbs at varying concentrations were added to cultures to assess the nature of the product recognized by the TLCs. mAb L243 recognizes a public epitope on HLA-DR molecules (23) and was obtained from the American Type Culture Collection. mAb Genox 3.53 recognizes DQw1 molecules (24) and was also obtained from the American Type Culture Collection as was mAb W6/32, which recognizes HLA class I molecules (25). mAb B7/21 recognizes a monomorphic HLA-DP allodeterminant (26) and was obtained from Ian Trowbridge (through the Salk Institute Cell Bank, La Jolla, CA). For blocking, stimulator LCLs were irradiated (10,000 rads) and plated at 2.5×10^4 cells per well in supplemented medium. mAbs were added at 50 μg/ml; this was followed by TLCs at 1×10^4 cells per well. The conditions of incubation and assessment of proliferation were as described above.

Paraformaldehyde Fixation. Paraformaldehyde was freshly diluted to 0.01% in sterile normal saline and adjusted to pH 7.2 with HCl. LCLs (2×10^6) were aliquoted into 12 × 75 mm sterile test tubes and pelleted at 200 × *g* for 10 min. Supernatant was aspirated, the pellet was resuspended, and 1 ml of 0.01% paraformaldehyde was added and incubated at room temperature for 20 min. Cold (4°C) unsupplemented RPMI 1640 medium containing 10% fetal calf serum was used to wash the fixed cells three times. Cells were finally resuspended at appropriate concentrations in supplemented medium containing 10% human plasma.

RESULTS

Alloantigen Specificities of T-Cell Clones. To determine the alloantigenic fine specificities of the anti-HLA-DR1 TLCs used in these studies, their proliferative patterns were analyzed on a panel of 115 homozygous LCLs as part of the Tenth International Histocompatibility Workshop (hereafter referred to as the workshop; ref. 22). Summarized representative data are presented in Fig. 1, where each of four TLCs has a unique specificity. In addition to DR1 and despite limiting the priming conditions, where only the DR1 molecule should have been recognized, significant recognition of LCLs bearing what were previously thought to be distinct class II antigens (e.g., DR4 and DRw6) was observed. In all, 59 TLCs were analyzed and those that recognized DR1 exclusively were found to be exceptional. It is possible that such heterogeneity might arise from (*i*) the recognition of other class II isotypes, (*ii*) cross-reactive epitopes shared by various DR molecules, or (*iii*) the recognition of minor antigens or peptides that might be associated with DR proteins (27). By using mAbs specific for DR, DQ, or DP in the workshop blocking assays, all four TLCs were blocked by the anti-DR mAb, L243, and not by anti-DQ and anti-DP antibodies (Fig. 2). Furthermore, if epitopes are shared among class II molecules, it might be expected that correlations of allelic DR amino acid sequences should explain clonal specificity patterns; for the TLCs in this study, no complete correlations could be found (data not shown). Alternatively, shared epitopes could arise from shared secondary, tertiary, and quaternary structures (molecular topography) somewhat independently of the alloantigen's primary structure.

Antigen Presentation of $HA_{306-320}$. Homozygous LCLs expressing the DR1;Dw1 specificities were used as antigen-presenting cells (APCs) to present peptide $HA_{306-320}$ to TLC HA1.7, which has previously been shown to be restricted by DR1$^+$ APCs (14, 15). Peptide at 0.1 μg/ml was added to DR1$^+$ (workshop identification no. 9006) irradiated LCLs at

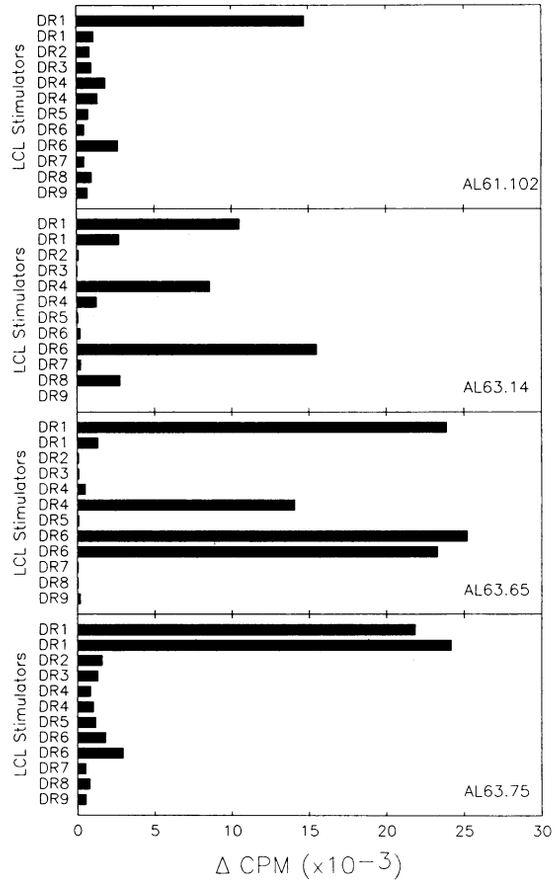

FIG. 1. Specificity profiles of four alloreactive T-cell clones. Allospecificity was measured in 2-day proliferative assays where 1×10^4 TLC cells were combined in triplicate with 2.5×10^4 homozygous LCLs from the workshop panel. Background cpm of each LCL in medium were subtracted.

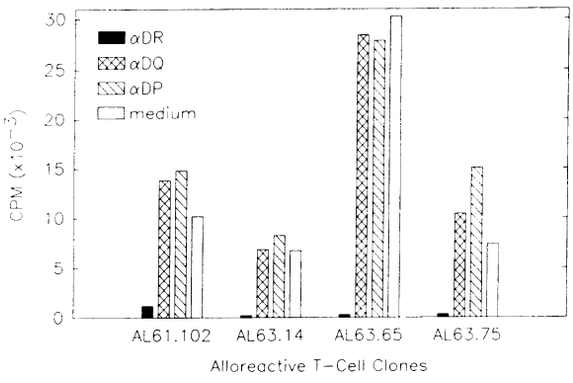

FIG. 2. Isotype-specific mAb blocking of alloreactive responses. mAbs were added at 50 μg/ml to optimized cultures of TLCs with LCL 9006. Proliferation was then measured. mAbs used were L243 (αDR), Leu10 (αDQ), and B7/21 (αDP).

2.5×10^4 cells per well. TLC HA1.7 was added at 1×10^4 cells per well and proliferation was measured as described above (Table 1). Strong clonal proliferation was observed when TLC and peptide were combined in the presence of LCL. Thus, peptide $HA_{306-320}$ is able to bind to and be presented by DR1;Dw1 APCs and subsequently recognized by the T-cell clone.

Peptide-Mediated Competitive Inhibition of Alloreactive TLC Proliferation. To examine the influence of the DR1-restricted $HA_{306-320}$ peptide on the recognition of DR1-specific, alloreactive TLCs, competitive inhibition experiments were performed. The proliferative response of the TLCs (1×10^4 per well) was determined while peptide concentrations were held constant at 30 or 100 μg/ml, and the number of LCL stimulator cells was increased (range, 2.5×10^4 to 20×10^4 cells per well). No effect was observed when LCLs were preincubated with the control ragweed peptide

Table 1. Antigen presentation of $HA_{306-320}$ to TLC HA1.7

TLC HA1.7*	LCL 9006†	$HA_{306-320}$, 0.1 μg/ml	Response,‡ cpm
+	+	+	18,655 ± 794
+	+	−	574 ± 34
+	−	−	112 ± 7
+	−	+	160 ± 33
−	+	−	385 ± 22

*DR1-restricted, $HA_{306-320}$-specific T-cell clone at 1×10^4 per well.
†Workshop identification number of DR1 LCL used as APC at 5×10^4 per well.
‡Triplicate cultures (mean cpm ± SEM).

RA_{51-65} (data not shown), which is also restricted by HLA-DR1 (18).

Three patterns of modulation were observed with $HA_{306-320}$ (Fig. 3 *a–d*). The first pattern, exemplified by TLC AL63.14 (Fig. 3*a*), included five of eight T-cell clones that were *inhibited* to varying degrees by the presence of increasing concentrations of the $HA_{306-320}$ peptide. At an LCL concentration of 1×10^4 per well, inhibition of clonal proliferation ranged from 38% to 86% at 100 μg of $HA_{306-320}$ per ml. Interestingly, rather than being inhibited by the $HA_{306-320}$ peptide, one T-cell clone, AL63.65, was *enhanced* 2- to 6-fold in its ability to proliferate in response to allostimulation (Fig. 3*b*). It is noteworthy that AL63.65 is not stimulated by peptide $HA_{306-320}$ and autologous APCs; additionally, peptide $HA_{306-320}$ bound to the original priming $DR1^+$ LCL induced the same enhancement (data not shown). A contrasting pattern was observed with the clones AL61.102 (Fig. 3*c*) and AL63.75 (Fig. 3*d*), which were not inhibited at all by $HA_{306-320}$.

Effects of Paraformaldehyde on $HA_{306-320}$-Mediated Enhancement of Proliferation by TLC AL63.65. To determine if enhancing and inhibitory effects of the peptide were due to peptide interactions with the DR1 molecule and not to some secondary effect on class II expression, paraformaldehyde

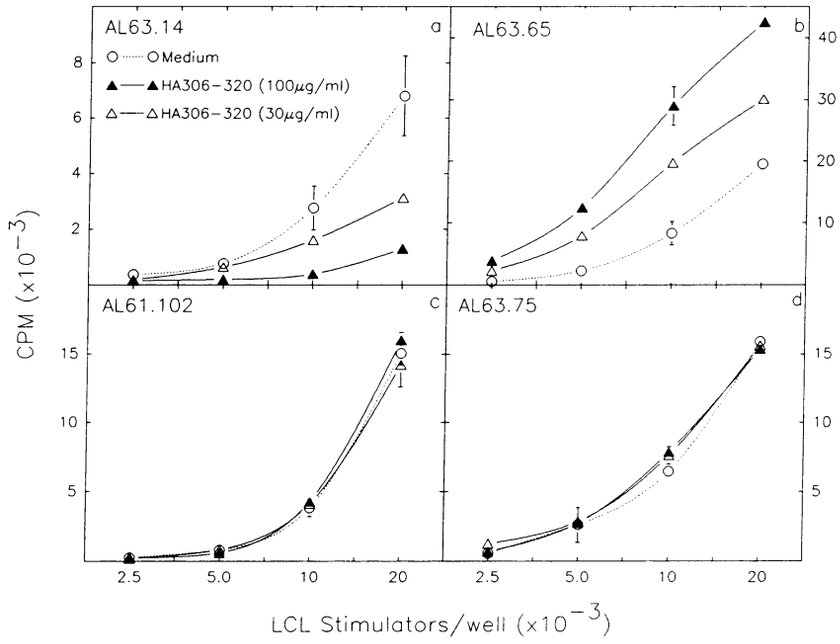

FIG. 3. Peptide-mediated modulation of alloreactive T-cell clones. HLA-DR1-specific alloreactive T-cell clones were derived by limiting dilution as described (19). A $DR1^+$ lymphoblastoid cell line, known to stimulate all of the clones, was irradiated (10,000 rads) and plated in microcultures (200 μl) at concentrations ranging from 2.5×10^3 to 2×10^4 per well. Negative controls consisted of LCL alone and clone alone in tissue culture medium supplemented with 10% pooled human plasma. $HA_{306-320}$ was added at 30 and 100 μg/ml to the lymphoblastoid cell line and incubated 2 hr at 37°C in fully humidified 5% CO_2. Alloreactive clones were then added at 1×10^4 per well, followed by incubation for 48 hr, at which time each microwell was pulsed overnight with 1.0 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol). Radiolabel incorporation was measured by liquid scintillation spectroscopy and expressed as the mean cpm of triplicate cultures (±SEM).

was used to fix stimulator cells before and after addition of peptide. Paraformaldehyde fixation of APCs prior to addition of peptide failed to alter the response of the antigen-specific TLC HA1.7 to $HA_{306-320}$ added at concentrations ranging from 0.1 to 100 μg/ml (Fig. 4). In contrast, enhancement of *alloreactive* TLC AL63.65 was completely abrogated if peptide was added after paraformaldehyde fixation, although, if peptide was added 4 hr *prior* to fixation, enhancement of TLC AL63.65 responses was observed (Table 2).

DISCUSSION

Our experiments were aimed at determining the differential effects of a DR1-binding HA peptide (residues 306–320) on DR1-restricted, alloreactive T-cell clones with different fine specificities. We found that $HA_{306-320}$ was able to modulate clonal responses, blocking most, but not all, alloreactive TLCs. Furthermore, in one instance, $HA_{306-320}$ enhanced the response to alloantigen. Since recognition of antigen by the T cell requires the formation of specific molecular interactions between peptide antigens and MHC molecules prior to binding by the T-cell receptor, several parameters could influence the interactions of different T cells with the same MHC/peptide complex.

Class II molecules may be similar in their structure to class I MHC products (28), based on striking sequence homologies, functional studies using mutated class II genes, and the crystal structure of HLA-A2 class I proteins (4, 5). If class II proteins resemble class I, then the proposed antigen combining site would consist of two parallel α-helices overlying a β-sheet platform that is held away from the membrane and presented to T cells on top of two immunoglobulin-like domains. In crystals of HLA-A2, this site between the α-helices is occupied by electron-dense material, suggestive of a peptide antigen. Such a structure would place certain constraints on the interaction of peptide with MHC and on the interaction of the T-cell receptor with the MHC/peptide complex. The dimensions of the proposed combining site could accommodate peptides of varying length (seven or more amino acids; ref. 29) depending on their secondary structure. Thus $HA_{306-320}$ may occupy only part of the combining site and could, in fact, bind to different locations within the combining sites of allelic MHC products. Furthermore, different peptides would have different affinities for MHC molecules based on their sequences and secondary structures. This implies that the location of critical residues in the combining site of a given MHC molecule may differ for individual peptides, which may also influence the site and orientation of the bound peptide.

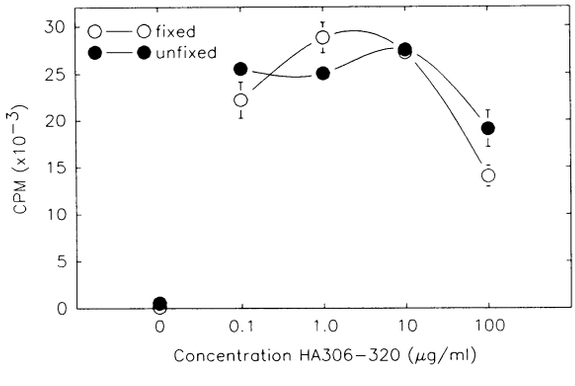

FIG. 4. Ability of paraformaldehyde-fixed and unfixed LCLs to present peptide to an antigen-specific TLC. $HA_{306-320}$ was added to fixed and unfixed cells (4×10^4 per well), followed by TLC HA1.7 at 1×10^4 per well. Proliferation was measured as described in the legend to Fig. 3.

Table 2. Effects of paraformaldehyde fixation on $HA_{306-320}$-mediated enhancement of proliferation by alloreactive TLC AL63.65

$HA_{306-320}$ addition*	Response,† cpm
None	15,661 ± 931
Prior to fixation	29,015 ± 752
After fixation	15,081 ± 1734

*$HA_{306-320}$ (100 μg/ml) was incubated 4 hr at 37°C with LCL9006 before or after fixation with 0.1% paraformaldehyde followed by washing three times.

†Alloreactive TLC was plated at 1×10^4 per well, and LCL 9006 was plated at 4×10^4 per well in triplicate cultures (mean cpm ± SEM).

Once the MHC/peptide complex is formed, binding of the T-cell receptor could be influenced by its affinity for the complex or the location to which it binds. If high affinity leads to an increased propensity for cross-reactivity as it does in antibody/antigen interactions, then high-affinity T-cell receptor interactions with MHC/peptide complexes could lead to a spectrum of cross-reactive combinations. T-cell receptors also need not bind the entire antigenic surface of the MHC/peptide complex nor with the same relative orientation, and thus different clones may express receptors that are specific for minutely distinct regions. The observation that not all clones are modulated identically may reflect variations in clonal specificities determined by the T-cell receptor or the failure of $HA_{306-320}$ to displace endogenous peptide. We argue that the combined effects of these two mechanisms may account for the extensive heterogeneity of T-cell responses to alloantigens (27).

The hypothesis that T-cell receptor and peptide might effectively compete for spatially related sites on the MHC molecule has been examined in experiments demonstrating that 4% of $I\text{-}A^d$-specific T cells were inhibited upon GAT binding to the $I\text{-}A^d$ molecule (13). We have extended these observations and show that a majority (62%) of human alloreactive T-cell clones is inhibited by the addition of $HA_{306-320}$. These results can be explained if $HA_{306-320}$ is able to displace endogenous peptides recognized by the alloreactive TLCs.

Since we have shown that our alloreactive TLCs have different fine specificities, some may recognize portions of the antigenic surface of the MHC/peptide complex that are not altered by the binding of $HA_{306-320}$. Alternatively, the presence of peptide may be irrelevant for certain T-cell receptors to bind, as seen in the failure of $HA_{306-320}$ to modulate clones AL61.102 and AL63.75. Finally, the $HA_{306-320}$ could simply fail to displace a subset of high-affinity endogenous peptides, which when complexed with MHC molecules would normally stimulate these TLCs.

Enhancement may derive from the formation of higher-affinity interactions between T-cell receptor and the MHC/peptide complex. Therefore, if the DR1/$HA_{306-320}$ complex more closely resembles the immunizing alloantigenic structure recognized by TLC AL63.65, then enhancement may result from the ability of $HA_{306-320}$ to bind at higher affinity than some endogenous peptides and so lead to the formation of a greater number of antigenic complexes than would otherwise occur without $HA_{306-320}$. Differential binding of the $HA_{306-320}$ peptide to DR1 molecules could arise from its varied success in competing for the combining site with endogenous peptides of varying affinities for DR1—that is, a low-affinity (relative to $HA_{306-320}$) endogenous peptide could be displaced unlike a high-affinity endogenous peptide. The observation that paraformaldehyde fixation abrogates enhancement suggests that internalization of the peptide is required. This cannot be attributed to a modification of the DR1 molecule since paraformaldehyde-fixed cells can pre-

sent peptide $HA_{306-320}$ with the same efficiency as unfixed accessory cells (Fig. 4). In the absence of internalization, complexes can form between $HA_{306-320}$ and DR1, but the number may be limited by the peptide-binding kinetics (11, 12). However, in the acidic environment of the endosomal compartment the dissociation rates of endogenous peptides and local concentrations of the exogenous peptides might lead to effective competitive inhibition under such conditions. It is noteworthy that AL63.65, which is specific for DR1, does not have heteroclitic specificity for $HA_{306-320}$ presented by autologous accessory cells, a phenomenon that is distinctive to that described by Heber-Katz and colleagues (16), nor does this reflect simple cross-reactivity between alloantigens and MHC-presented foreign antigens (6). Although the molecular mechanisms have not been defined, if enhancing phenomena are commonplace, then caution should be exercised in designing therapies based on the premise that binding of a high-affinity ligand to its specific MHC molecule will always function to inhibit T-cell recognition of another antigen (30).

The different patterns of observed modulation suggest that T-cell recognition of a particular class II alloantigen is comprised of a heterogeneous collection of clonal specificities. In addition, it is important to emphasize that these effects can be influenced by the affinities and specificities of the molecular interactions between T-cell receptors and the MHC/peptide complex as well as the differential binding of endogenous peptides. This may begin to explain the observation that a large proportion of T cells apparently recognizes allogeneic MHC molecules (27). The diversity of specificity we and others have observed (17, 31) contrasts with the view that allorecognition involves MHC molecules alone and could reflect the potentially large number of endogenous peptides that may occupy the MHC class II combining site as well as suggesting that many different T-cell receptors recognize the same MHC/peptide complex. If appropriate peptides can be designed, then peptide-mediated modulation of T-cell recognition may prove valuable in novel approaches to transplantation biology and the regulation of autoimmunity.

We thank Tom Sell, Roxanne Pinzl, and Joe Guckenberger for technical assistance and Drs. Don Wiley and Peter Parham for stimulating discussions. These experiments were supported by National Institutes of Health Grant AI22832 (D.D.E.) and the Harry and Lynde Bradley Senior Scholars in Biomedicine program of the Blood Center Research Foundation (J.R.L.). D.D.E. is supported by Research Career Development Award AI00799.

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