Cytotoxic T lymphocytes recognize a reconstituted class I histocompatibility antigen (HLA-A2) as an allogeneic target molecule

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ABSTRACT Recent findings suggest that peptide fragments of newly synthesized proteins may associate intracellularly with nascent chains of class I histocompatibility antigens (termed MHC-I proteins because they are encoded by genes of the major histocompatibility complex) and that these peptide adducts may be required for the folding or stability and perhaps even the transport of these proteins to the cell surface. To determine whether these proteins can be reconstituted from their separated subunits into ostensibly native molecules in the absence of added peptides, we denatured a purified human MHC-I protein (HLA-A2) with 4 M NaSCN, separated its heavy (α) and light (β_2 -microglobulin) chains by gel filtration, and then mixed them in the presence of a 3-fold molar excess of β_2 -microglobulin and absence of added peptides. The reconstituted protein, recovered in 10% yield, was indistinguishable from native A2 in its reactivity with a monoclonal antibody (BB7.7) and its ability to specifically activate A2-specific CD8⁺ T cells. Inasmuch as the reconstituted A2 contained no detectable peptide adducts (we estimate <1 per 100 on a molar basis, assuming peptides of 2–5 kDa), the results suggest that peptide-free A2 can be recognized by CD8⁺ T cells and that peptide adducts are not essential for the MHC-I protein to maintain an ostensibly native structure.

Considerable evidence suggests that the antigenic structures recognized on target cells by CD8⁺ cytotoxic T lymphocytes (CTLs) are binary complexes formed by the association of peptide fragments of protein antigens with class I histocompatibility antigens (encoded in the major histocompatibility complex and termed MHC-I proteins). How and when peptide fragments become associated with MHC-I proteins, and what proportion of the MHC-I molecules on a cell's surface are associated with peptides, are unresolved questions. However, x-ray crystallographic analysis of a human MHC-I protein (HLA-A2) has suggested that virtually all of the peptide binding sites on the purified protein are occupied by peptides (1). Moreover, it has been observed that an MHC-I protein which was synthesized but not expressed on the surface of a mutant cell line could be induced to appear on the cell surface simply by incubating the cells with a peptide that associates with that MHC-I protein (2). This finding has raised the possibilities that peptide fragments of newly formed proteins might normally become associated intracellularly with nascent MHC-I polypeptide chains as they fold into mature MHC-I molecules and that the peptide adducts may even be required for the folding and stability of class I MHC molecules and for their ultimate transport to the cell surface.

To help evaluate those possibilities, it would be useful to know whether or not the constituent heavy and light chains of an MHC-I protein can be separated and then reassociated

in the absence of added peptides to form stable, native protein molecules. In an earlier attempt to meet this objective, it was found that when the subunits of HLA-A2 were separated in a denaturing solvent they could reassociate after the denaturing solvent was removed, but not if the subunits were first separated from each other—e.g., by gel filtration (3). These observations have been regarded, retrospectively, as evidence in support of the idea that peptides may be required for the folding and association of the subunits of MHC-I proteins. Here we describe another approach to the reconstitution of native HLA-A2 from its separated heavy and light chains. When we denatured the protein, separated its polypeptide chains, and then recombined them in the presence of excess light chains [β_2 -microglobulin (β_2 m)] and in the absence of exogenous peptides, the heterodimeric protein that formed was not distinguishable from native A2 by monoclonal antibodies or by CTLs that are specific for HLA-A2. If the reconstituted HLA-A2 molecules are free of peptides, as we surmise from preliminary analyses, these findings indicate that a stable MHC-I heterodimer can be formed in the absence of peptide adducts and that “naked” MHC-I molecules can be recognized by some (alloreactive) CTLs.

MATERIALS AND METHODS

Purification of HLA-A2. HLA-A2 was isolated as described (4) and purified further by HPLC gel filtration (Beckman/Altex Sepherogel TSK 3000SW column, 0.75 × 30.0 cm).

Renaturation of HLA-A2. A2 was denatured by incubating it for 30 min at room temperature in 4 M NaSCN (pH 7.3). Low molecular weight material was then removed by gel filtration (TSK 3000SW column in 4 M NaSCN) or membrane filtration using a Centricon-30 (Amicon) filtration unit. Human β_2 m (Calbiochem) was further purified by ion-exchange chromatography [TSK DEAE 5 PW equilibrated with 0.01 M Tris-HCl (pH 8.0); elution with a linear 0–0.25 M NaCl gradient in 0.01 M Tris-HCl (pH 8.0) over 1 hr] before incubation with 4 M NaSCN for 30 min at room temperature. This denatured β_2 m was then mixed with the denatured A2's heavy and β_2 m chains so as to effect a final 3-fold molar excess of β_2 m over heavy chain, taking into account losses of A2-derived β_2 m during membrane filtration (where appropriate). After 10 min at room temperature, the thiocyanate was gradually removed by stepwise dialysis in a Centricon-10 filtration unit over a period of 1 hr; the final thiocyanate concentration was estimated to be <1 mM. The resulting renatured A2 was used without further purification or was isolated from excess β_2 m by gel filtration (TSK 3000 SW in 10 mM Tris-HCl, pH 7.4/150 mM NaCl).

Abbreviations: MHC-I, major histocompatibility complex class I; β_2 m, β_2 -microglobulin; CTL, cytotoxic T lymphocyte; IL-2, interleukin 2.

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Competitive Immune Cytolysis Assay. A2 and renatured A2 were assayed for their ability to inhibit immune cytotoxicity of ^{51}Cr -labeled JY cells (A2.2; B7.7) by a cytotoxic monoclonal antibody, BB7.7, as described (4). Percent specific lysis was calculated as $100(a - b)/(c - b)$, where a is experimentally released ^{51}Cr , b is spontaneously released ^{51}Cr (i.e., in absence of antibody and complement), and c is the total amount of ^{51}Cr released by adding Nonidet P-40 (0.25%) to the labeled cells. Nonspecific lysis by complement in the absence of antibody was always $<2\%$ of c . Results are expressed as the percent inhibition of maximum lysis, which is defined as percent specific lysis in the absence of inhibitor.

SDS/PAGE and Autoradiography. SDS/PAGE was performed using 18% acrylamide gels containing 6 M urea (5). Gels were stained with silver (6) or with Coomassie brilliant blue R (Sigma). Samples containing 4 M NaSCN were desalted using disposable reverse-phase C_{18} cartridges (Sep-Pak; Waters). Where appropriate, SDS/polyacrylamide gels were dried onto Whatman 3MM chromatography paper and exposed to Kodak X-Omat AR x-ray film for 24–72 hr.

Iodination. Samples in phosphate-buffered saline [PBS: 10 mM sodium, potassium phosphate (pH 7.4) containing 150 mM NaCl] were radioiodinated with 1 mCi (37 MBq) of carrier-free Na^{125}I in the presence of solid-phase *N*-chlorobenzenesulfonamide (Iodo-Beads; Pierce) (7).

CTLs. A human alloreactive CTL line (termed A2p) specific for HLA-A2 was originally isolated from a mixed lymphocyte culture of JY lymphoblastoid stimulator cells (A2.2; B7.7; DR4,6) and peripheral blood mononuclear cells from donor HNE (A1,3; B35,38; DR4,6; DQw1,w3) by their adsorption to purified A2 that was adsorbed onto plastic dishes (8). Cultured A2p cells were CD8^+ and highly cytolytic for A2^+ cells.

T-Cell Activation Assay. Sterile flat-bottomed 96-well tissue culture plates (Costar) were coated with native, renatured, or denatured HLA-A2 or with monoclonal antibody OKT3 in PBS overnight at 4°C . Unadsorbed A2 was removed and wells were "blocked" with PBS containing 1% bovine serum albumin. Stationary-phase A2p cells (2×10^5 per well)

in serum-free medium (AIM V; GIBCO) containing interleukin 2 (IL-2, 20 units/ml) were added and the plates were incubated at 37°C in a 5% CO_2 atmosphere. When JY cells were used as stimulators in lieu of adsorbed A2, 3.3×10^4 irradiated (6000 rads; 1 rad = 0.01 Gy) cells in AIM V (with recombinant IL-2 at 20 units/ml) were added per culture. After 2, 4, or 6 days, 1 mCi of $[^3\text{H}]$ thymidine (NEN) was added per well, and the cells were harvested the next day onto fiberglass filters (Skatron, Sterling, VA). Incorporated $[^3\text{H}]$ thymidine was measured in a Beckman β counter. The stationary-phase A2p cells used in the assay were taken 14–21 days after stimulation with irradiated JY cells; at this time they showed no sign of proliferation by cell count.

RESULTS

Reversible Denaturation of HLA-A2. To promote the dissociation of peptide adducts from purified HLA-A2 (Fig. 1a and Fig. 2, lane a), the protein's heavy (α) and light ($\beta_2\text{m}$) chains were dissociated by incubating A2 in 4 M NaSCN at neutral pH and room temperature. Gel filtration of the resulting mixture was performed in the presence of 4 M NaSCN and fractions were monitored by absorbance at 280 nm. The single A2 peak in Tris buffer at pH 6.8 (peak I, Fig. 1a) collapsed in 4 M NaSCN to two major peaks (Fig. 1b) that were eluted at ≈ 35 kDa (peak II) and 12 kDa (peak III) and two minor peaks (IV and V) that were eluted close to the inclusion volume. The major peaks II and III corresponded to heavy (α) chain and $\beta_2\text{m}$, as shown by their mobility in SDS/PAGE (see below for Fig. 2, lanes b and c and lanes d and e, respectively). No evidence of $\alpha/\beta_2\text{m}$ heterodimers was found in the leading edge of the α -chain peak (Fig. 2, lane b), indicating that chain separation was complete. An identical chromatogram was obtained when A2 was denatured in 8 M guanidine hydrochloride (1 hr at 37°C) and the gel filtration fractions were monitored by absorbance at 215 nm (data not shown).

The α and $\beta_2\text{m}$ peak fractions were collected and added back to one another in the presence of additional purified human $\beta_2\text{m}$ (in 4 M NaSCN), sufficient to effect a 3-fold molar

FIG. 1. Gel filtration chromatograms. (a) Native A2 in 10 mM Tris-HCl (pH 6.8). (b) Denatured A2 in 4 M NaSCN. (c) The mixture present after renaturation of A2 in the presence of excess $\beta_2\text{m}$. (d) Peak VI from c in 10 mM Tris-HCl (pH 6.8). Retention times (RT) for peaks I–VIII are given at right. The column (TSK 3000SW) was precalibrated with a mixture of bovine serum albumin (68.0 kDa; RT, 25.6 min), hen egg white ovalbumin (42.7 kDa; RT, 28.4 min), and horse heart cytochrome c (12.4 kDa; RT, 33.0 min).

FIG. 2. SDS/PAGE analysis of peaks shown in Fig. 1. Purified A2 (peak I, Fig. 1) yields two bands (lane a) corresponding to α chain (≈ 35 kDa) and β_2 m (≈ 12 kDa). Samples were taken from the leading edge (lane b) and trailing edge (lane c) of peak II (Fig. 1b) and the leading edge (lane d) and trailing edge (lane e) of peak III (Fig. 1b). Lanes a-e were silver-stained. Samples of peaks VI, VII, and VIII (Fig. 1c and d) were run in lanes f, g, and h, respectively, and protein was detected by Coomassie blue. Molecular mass markers (kDa) are shown to the left of lanes a and f.

excess of β_2 m over the α chain. Excess β_2 m was added to drive the renaturation equilibrium in the direction of α/β_2 m heterodimer formation. After removal of NaSCN, the final mixture was analyzed by gel filtration (Fig. 1c). Two peaks were evident: one (peak VI) corresponding to the α/β_2 m heterodimer and the other (peak VII) to free β_2 m (see Fig. 2, lane f for peak VI and lane g for peak VII). The identity of the first peak (VI) was verified by a further gel filtration run, which resulted in a monodisperse preparation consisting of α/β_2 m heterodimers (peak VIII, Fig. 1d and Fig. 2, lane h). The latter represents reassociated A2 and is expected to be virtually free of peptide adduct, since material of <10 kDa was removed following denaturation (Fig. 1b) and was absent during the renaturation process. In other preparations low molecular mass material was removed in the continuous presence of NaSCN by membrane filtration, which proved to be as effective as gel filtration.

The average yield of renatured α chains from eight such experiments was $10 \pm 1.9\%$. When excess β_2 m was omitted from the renaturation mixture, the average yield fell to below 2%.

To address the possibility that some α chain may have retained its peptide adduct through the denaturation procedure, we determined the sensitivity of the silver-stain technique used to analyze α chain by SDS/PAGE. We could detect as little as 1–5 ng of a peptide in the molecular mass range 2.5–16 kDa. Since 5–10 μ g of α chain was applied to lane b of Fig. 2, and no silver-stained material was observed in the range 2–15 kDa, it appeared that peptide was present at <1 part per 1000–10,000 by weight or less than around 1 per 100–1000 in molar terms (for a peptide of 3.5 kDa).

The two minor peaks that appeared reproducibly following denaturation (peaks IV and V in Fig. 1b) could have represented the electron-dense material seen in the crystal structure of A2, thought to be specifically bound peptides (1). Buus *et al.* (9) reported the release of low molecular mass material denatured from I-A^d; though too little was recovered to be characterized chemically, it contained immunologically active peptides by bioassay. Accordingly, we radiolabeled with 125 I the low molecular mass material released from 250 μ g of A2 following denaturation in 4 M NaSCN (Fig. 1b, peaks IV and V). The radiolabeled material was analyzed by SDS/PAGE followed by autoradiography. Fig. 3, lane a, clearly shows a band migrating at ≈ 2 kDa; also evident is some β_2 m (12 kDa), which passed through the membrane

FIG. 3. Autoradiographs of SDS/PAGE analysis of 125 I-labeled low molecular mass material isolated from denatured A2 (lane a), mock-denatured A2 (PBS in lieu of 4 M NaSCN) (lane c), and renatured A2 that was subjected to a second round of denaturation (lane d). Gel filtration of the mixture in lane a enabled the isolation of low molecular mass material (lane b). Molecular mass markers (kDa) are shown to the left of lanes a, b, and d.

used to separate high and low molecular mass material (see *Materials and Methods*). The low molecular mass material was separable from β_2 m on a Sephadex G-50 column (Fig. 3, lane b), and it was eliminated by incubating it overnight at 37°C with either trypsin at pH 7.3 or pepsin at pH 2.5, indicating that it was peptide in nature. It was not released from A2 when NaSCN was replaced by PBS in a mock denaturation (Fig. 3, lane c); it is especially notable that it was not detected when renatured A2 was itself denatured in 4 M NaSCN and fractionated by membrane filtration (Fig. 3, lane d), though the β_2 m band was again clearly visible, as expected.

Reactivity of Renatured A2. To determine whether the reassociated α and β_2 m chains (renatured A2) had regained their native configuration, we made use of monoclonal antibody BB7.7, a cytotoxic antibody that recognizes a monomeric combinatorial determinant formed by α and β_2 m chains in association (10). Accordingly, the ability of renatured A2 to inhibit immune cytotoxicity of JY cells (A2,2) by BB7.7 plus complement was tested. Fig. 4a shows that native A2 at a concentration of 35 nM (1.75 μ g/ml) caused 50% inhibition of JY lysis (under conditions where antibody is limiting). In negative controls, the lysis of JY cells was not inhibited by free β_2 m or ovalbumin. Fig. 4b shows that purified, renatured A2 effects 50% inhibition at 1.50 μ g/ml, identical to native A2 (within experimental error, ± 0.5 μ g/ml). Furthermore, when the final renaturation mix (peaks VI and VII in Fig. 1c) was used in the assay, the 50% inhibition point was around 3 μ g/ml, which was consistent with the concentration of renatured A2 in the mix as judged from protein assays of the two separate peaks. These results indicate that the presence of excess β_2 m in the assay did not affect accurate determination of renatured A2 in the mixture, and subsequent yields of renatured A2 were calculated from inhibition data of the final renaturation mixture. In contrast, no inhibition was seen with denatured A2 (heated to $\approx 100^\circ\text{C}$ in the presence of 5 mM dithiothreitol and then alkylated with iodoacetamide). The α chain that failed to renature was evidently lost as precipitate or by adsorption to vessel walls, since the final renatured mixture contained only renatured A2 and excess β_2 m (Fig. 1c).

Recognition of Renatured A2 by Alloreactive CTLs. We have previously shown (8) that an alloreactive anti-A2 CTL cell line derived from an A2-negative individual specifically binds and responds to purified A2 adsorbed to plastic. Designated as A2p cells and maintained in culture for many months, they are $>95\%$ CD8⁺ and lyse human A2-bearing target cells. They also lyse mouse cells that have been coated

FIG. 4. Assay for A2 based on inhibition of complement-dependent cell lysis. (a) Inhibition of lysis of ^{51}Cr -labeled JY cells by soluble, native A2 (●), β_2m (○), and ovalbumin (□). (b) Inhibition of lysis of ^{51}Cr -labeled JY cells by serial dilutions of denatured A2 (○), crude renatured A2 corresponding to a mixture of peaks VI and VII in Fig. 1c (■), and purified renatured A2 corresponding to peak VIII of Fig. 1 (●). In both a and b, the maximum response was 42% specific lysis of JY.

with purified, papain-solubilized native A2, but not when coated with denatured A2 (8).

To determine whether these CTLs could recognize renatured A2 adsorbed to plastic plates, we used [3H]thymidine incorporation to measure their specific activation. For this purpose, A2p cells were cultured in the presence of recombinant IL-2 in 0.5-cm tissue culture wells that had been coated with either native A2, renatured A2, or A2 that had been denatured by reduction and alkylation with iodoacetamide. In addition, as positive controls, cultures were set up in wells coated with a monoclonal antibody (OKT3) to the CD3 component of the T-cell antigen-specific receptor complex. Proliferative responses were detected on day 2 and day 6 and were maximal on day 4. Fig. 5 shows dose-dependent responses of A2p cells that had been incubated for 4 days in microtiter wells coated with various concentrations of OKT3, native A2, and renatured A2. The maximum stimulation index observed with OKT3 was 4.4 in the experiment shown; in other experiments, using different preparations of OKT3, responses up to 27.0 were seen. The responses to renatured A2 and native A2 were indistinguishable at all coating concentrations tested. No response was elicited by ovalbumin or by denatured A2. The natural targets for A2p cells, JY cells, were also tested as a source of stimulation at a CTL/target cell ratio of 3:1 (chosen to provide roughly the same number of A2 molecules per culture as wells coated with 50 μl of A2 at 5 $\mu g/ml$, assuming a coating efficiency of 0.1%). On day 4, the stimulation index for A2p cells cultured with irradiated (6000 rads) JY cells was 4.1 ± 0.1 after correction of the [3H]thymidine incorporation for uptake by target cells alone. Thus, under the experimental conditions used, renatured A2 and native A2 were approximately as effective as JY cells in stimulating A2p cells. In all cases, the proliferative response was found to be dependent on the addition of IL-2.

Although the observed dose-responses of A2p cells to native A2 and renatured A2 were indistinguishable, it was possible that some CTLs responded only to renatured A2 and others only to native A2. To test this possibility, two 100-fold dilutions of a mixture of native A2 and renatured A2, each at 50 $\mu g/ml$, were used to coat wells. If the observed responses were due to separate populations of CTLs, at each dilution the response to the mixture would approximate the sum of the responses to the individual components, and this effect would be especially notable at concentrations that gave maximal responses when either protein was used alone. Fig. 5 shows that this was not the case: the A2p cells' response to the

mixture was similar to their response to native A2 or renatured A2 individually, indicating that the same cells responded equally well to both forms of A2.

DISCUSSION

In this study we have shown that it is possible to dissociate the A2 heavy (α) and light (β_2m) chains in the presence of a

FIG. 5. [3H]Thymidine incorporation by 2×10^5 A2p cells after 4 days in culture with purified anti-CD3 antibody (OKT3) (●), ovalbumin (×), or native (○) or renatured (□) forms of HLA-A2 in the presence of recombinant IL-2 (20 units/ml). In addition, responses in equimolar mixtures of native A2 and renatured A2 are shown (●). Concentrations on abscissa refer to each protein individually and in the mixture.

chaotropic agent, remove low molecular weight material, and then reassociate the separated chains in the presence of excess β_2m . Around 10% of the α chains reassembled into α/β_2m heterodimers. The latter were indistinguishable from native A2 with respect to their reactivity with the monoclonal antibody BB7.7 and an anti-A2 alloreactive CTL line (A2p cells). Denatured A2, in contrast, was not recognized by either the antibody or the CTLs.

Our results suggest that stable α/β_2m heterodimers can be formed *in vitro* in the absence of bound peptides. These results would at first appear to contradict those of Townsend *et al.* (2), who suggested that $\alpha\beta$ assembly and transport to the cell surface *in vivo* are dependent upon bound peptide. They do not, however, preclude the possibility that $\alpha\beta$ assembly can be driven *in vitro* by the addition of excess β_2m in the absence of peptide. Indeed, we, and others (3), have been unable to renature HLA-A2 in the absence of excess β_2m . The ability to prepare renatured A2 that is ostensibly peptide-free enabled us to address the central question of allorecognition—i.e., whether CTLs that react specifically with allogeneic MHC-I proteins recognize peptide/MHC complexes or MHC molecules alone.

According to a modern version (11) of a hypothesis advanced about 12 years ago by Matzinger and Bevan (12), under physiologic conditions any particular MHC-I protein on a cell's surface exists as a highly heterogeneous population of binary complexes, each formed with one of the many peptides arising in that cell as a result of proteolytic degradation of newly synthesized cellular protein. Accordingly, an alloreactive T cell that is nominally specific for a particular MHC-I protein actually recognizes only one or a few of the multitude of different complexes formed by various peptides with that MHC-I protein on the target cell surface. The multitude of different complexes formed by a particular MHC-I protein could be recognized by many different T-cell clones.

However, it is also possible that a T cell can recognize an allogeneic MHC-I protein alone, and that the role of associated peptide adducts is to maintain the MHC-I molecule in a native conformation. Another possibility is that alloreactive T cells can recognize MHC-I molecules having no associated adducts—i.e., as “naked” molecules—even though MHC-I protein often exists as complexes with peptides. According to this possibility every molecule of an MHC-I protein encoded by the appropriate allele could be a ligand for alloreactive CTLs that are specific for that MHC-I protein (the determinant-density hypothesis, refs. 13 and 14).

Our experiments showed that renatured A2, judged to be free from peptide adducts (by the absence of releasable low molecular mass material following a second round of denaturation; Fig. 3, lane d), was as effective as native A2 in stimulating anti-A2 CTLs. Hence peptides appear not to be essential for allorecognition, at least for a subset of alloreactive CTLs. Moreover, under the experimental conditions used, both forms of A2 were as effective in stimulating A2p cells as was the “natural” ligand on the surface of JY cells, where it is indeed possible that a majority of A2 molecules contain bound peptides. Although the results obtained with intact JY cells and with purified A2 adsorbed on plastic are difficult to compare, they suggest that the presence of bound peptide in the natural MHC-I ligand neither ablates nor enhances its recognition by A2p cells.

The A2p cells were originally isolated from a mixed lymphocyte reaction according to their ability to recognize and respond to purified soluble A2 adsorbed to plastic (8). It is thus possible that A2p cells represent a subpopulation of alloreactive CTLs having an unusually high capacity to recognize determinants on A2 alone. This possibility is supported by the observation that not all anti-A2 alloreactivity

was removed from the mixed lymphocyte culture when A2p cells were originally isolated by adsorbing (“panning”) them on A2 (8).

An alternative explanation of our results is that the 10% of renatured HLA-A2 molecules recovered represented a population of α chains that had not released bound peptides during incubation with 4 M NaSCN, despite the effect of the denaturant in causing this chain and the β_2m subunit to dissociate: thus the recovered renatured A2 molecules would be expected to be enriched with nondissociating peptides. We think that this possibility is unlikely, since when α chains used for renaturation were heated to 100°C in the presence of 0.1% SDS and then analyzed by PAGE, no low molecular mass material could be visualized in a 5- to 10- μ g sample of renatured A2 with a sensitive silver staining technique that was capable of visualizing 1–5 ng of a homogeneous peptide of 2–16 kDa. This result suggests that any single retained peptide present as a contaminant in the α -chain preparations would have been present at a level below around 1 mol of peptide per 100–1000 mol of α chains (see *Results*). Moreover, if retained peptides were relevant for the specific response of CTLs to renatured A2, they would have to have been selectively retained (while other peptides were selectively lost), since the CTLs responded equally well to renatured A2 and native A2. Such a fortuitous selective retention seems implausible. Therefore, we believe our results support the determinant-density model of alloreactivity.

Though we here emphasize differences between the two models, it should be noted that they are not, of course, mutually exclusive; the ligands for some alloreactive CTLs might be binary MHC/peptide complexes (e.g., ref. 15), while for others they might be an MHC protein alone, regardless of whether or not it is complexed with peptide. Panning of alloreactive CTLs (in a mixed lymphocyte culture) on purified, adduct-free MHC-I protein, and on the same protein having the appropriate peptide adducts, may help determine which type of allorecognition is the more common.

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