Peptide-independent Recognition by Alloreactive Cytotoxic T Lymphocytes (CTL)

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Summary

We have isolated several H-2Kb–alloreactive cytotoxic T cell clones and analyzed their reactivity for several forms of H-2Kb. These cytotoxic T lymphocytes (CTL) were elicited by priming with a skin graft followed by in vitro stimulation using stimulator cells that express an H-2Kb molecule unable to bind CD8. In contrast to most alloreactive T cells, these CTL were able to recognize H-2Kb on the surface of the antigen processing defective cell lines RMA-S and T2. Furthermore, this reactivity was not increased by the addition of an extract containing peptides from C57BL/6 (H-2b) spleen cells, nor was the reactivity decreased by treating the target cells with acid to remove peptides bound to MHC molecules. The CTL were also capable of recognizing targets expressing the mutant H-2Kbm8 molecule. These findings suggested that the clones recognized determinants on H-2Kb that were independent of peptide. Further evidence for this hypothesis was provided by experiments in which H-2Kb produced in Drosophila melanogaster cells and immobilized on the surface of a tissue culture plate was able to stimulate hybridomas derived from these alloreactive T cells. Precursor frequency analysis demonstrated that skin graft priming, whether with skin expressing the wild-type or the mutant H-2Kb molecule, is a strong stimulus to elicit peptide-independent CTL. Moreover, reconstitution experiments demonstrated that the peptide-independent CTL clones were capable of mediating rapid and complete rejection of H-2–incompatible skin grafts. These findings provide evidence that not all allore cognition is peptide dependent.

Alloreactive T cells recognize foreign MHC molecules and mount vigorous responses either in vivo, leading to graft rejection, or in vitro during a mixed lymphocyte reaction. Several models for the specificity of alloreactive T cells have been proposed. Matzinger and Bevan (1) originally suggested that these T cells recognized antigens, now known to be peptides, bound to foreign MHC molecules. Various modifications of this theory have focused on whether the TCR contacts both the MHC molecule and the peptide or either the peptide or the MHC molecule only (1). In the latter case, although not involved in the contact with the TCR, the bound peptide profoundly influences the conformation of the MHC residues comprising the epitope (2, 3). An alternate model in which alloreactive T cells recognize MHC molecules in a peptide-independent fashion has also been hypothesized (4). In this scheme, T cells recognize the polymorphic differences of the foreign MHC I molecule in a peptide-independent fashion. It is further hypothesized that these peptide-independent determinants are expressed at high densities on the surface of antigen-presenting cells.

Evidence supporting the involvement of peptide in alloreactivity is provided by experiments in which CTL could kill the antigen processing defective cell lines, T2 and RMA-S, only after pulsing the targets with MHC-binding peptides isolated from syngeneic cells (5–8). In some instances, such as the anti H-2Ld allo clone 2C, the sequence of the allopeptide has been determined (9). In addition, stimulation with allogeneic APCs pulsed with a specific peptide, elicits peptide-specific alloreactive T cells (10). Further evidence for peptide specificity of alloreactive T cells are experiments demonstrating that some clones require peptides that are specific to certain tissues (7, 11) or species (12).

Although the above experiments have provided evidence that alloreactive T cells are peptide dependent, several observations are consistent with a peptide-independent ligand for some alloreactive T cells. A number of reports have described alloreactive T cells that can recognize the TAP transporter–defective cell lines, particularly if the target cells are cultured at 27°C (12–16). These findings are difficult to interpret in that, although these mutant cells are unable to efficiently transport peptides from cytosolic pro-
teins into the endoplasmic reticulum for loading into class I molecules, peptides from leader sequences and other proteins have been identified in MHC class I molecules on the surface of these cells (17–23). Therefore, it is possible that alloreactive T cells that react with these mutant APCs are specific for such peptides. In light of this explanation, the hypothesis that some alloreactive CTL recognize either empty MHC molecules or determinants that are unaffected by the bound peptide is not widely accepted.

In this present report, we show that some H-2Kb−alloactive CTL are peptide independent in their reactivity based on the: (a) ability to recognize H-2Kb−on the antigen presentation defective cell lines T2 and RMA-S, (b) inability of a peptide extract eluted from C57BL/6 spleen cells to enhance the killing of these antigen processing defective target cells; (c) failure of acid treatment of target cells to ablate CTL killing; (d) recognition of the mutant molecule H-2Kbm8−; (e) reactivity with immobilized H-2Kb−produced in a Drosophila melanogaster expression system, even after treatment with acid to remove any bound peptide. These peptide-independent CTL are enriched in the spleen after priming of the mice with a skin graft. Furthermore, injection of one of these CTL clones into a T cell-depleted mouse was sufficient to mediate rapid and complete skin graft rejection. The identification of peptide-independent alloreactive T cells, as well as the elucidation of an appropriate priming stimulus for their expansion and isolation is in contrast to the widely accepted hypothesis that all allore cognition is peptide dependent.

Materials and Methods

Mice. B10.D2 and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred to produce (BALB/c × B10.D2) F1 (referred to herein as CD2F2) progeny. 28.1 mice are transgenic for the H-2Kb−gene on a B10.D2 background. 29.7 mice are transgenic for the mutant (non-CD8 binding) H-2Kb−gene on a B10.D2 background (24).

Target Cell Lines. M12.C3 (H-2b−) (25) or the peptide processing defective T2 (17, 18, 21, 26, 27) and RMA/S (28-30) cells expressing the H-2Kb−class I molecules was generated by transfecting an exon shuffled MHC class I gene encoding the H-2Kb−α1 and α2 domains and the H-2Dd−α3 domain as previously described (31). These cells are referred to herein as either M12.Kb or T2.Kb−T1 cells, which are the non-mutant parental cells from which T2 were derived (17), were also transfected with H-2Kb−Dd−gene.

Generation of T Cell Clones and Hybridomas. All T cell clones described were isolated by limiting dilution from the spleens and lymph nodes of CD2F2 mice that had been engrafted with trunk skin from a 29.7 strain transgenic mouse. Lymph node and spleen cells were harvested on day 14 after engraftment and stimulated in vitro with irradiated 29.7 spleen cells. The clones were also restimulated weekly with irradiated 29.7 stimulator spleen cells. Responder and stimulator cells were co-cultured in DMEM/FC/S(10%) containing Con A −stimulated rat spleen supernatant (T cell medium). All clones characterized in this study were confirmed, by flow microfluorimetry analysis, to be CD8−/αβ−T cells (data not shown). The alloreactive H-2Kb−CTL clone 4.1 was generated by limiting dilution cloning from a primary M LR of B10.D2 stimulated with C57BL/6 spleen cells. The ovalbumin-reactive H-2Kb−restricted CTL clone B3 was obtained from Dr. M. Bevan and was maintained as described (32). Hybridomas were developed as described (32). In brief, 5 × 107 clonal T cells were fused to 108 BW 3.3/CD8−cells (a derivative of the BW S147 line transfected with a β-galactosidase reporter gene behind the IL-2 promoter (33) in 1 ml of MEM/PEG-1540 (1:1 wt/vol) for 1.5 min and then slowly diluted in 50 ml of MEM. Cells were then washed 1 × in HBSS and then diluted in 100 ml of MEM/FC/S and aliquoted into 96-well plates. HAT was added 24 h later and cells were refed weekly with 1 × HAT-containing medium. Colonies were picked from the 96-well plates and grown up in 24-well plates in HAT containing medium before screening for reactivity. Selected hybridomas were maintained in DMEM/FC/S.

A say of Cytotoxicity. Cytotoxic activity was measured in a standard 51Cr-release assay. Target cells were labeled with 51Cr (100 μCi/106 cells) for 1.5 h at 37°C. For treatment with acid the 51Cr-labeled target cells were suspended in a citric phosphate solution (0.131 M citric acid, 0.066 M sodium phosphate, 1% BSA) at pH 3.0 (34). M12 cells were incubated in acid for 15 min, and T2 cells for 8 min, at room temperature. They were recovered by centrifugation and resuspended in DMEM/FC/S. After this treatment, the viability of the cells was generally around 75% as assessed by trypan blue exclusion. Labeled target cells (5000 per well), were added to each well of a V-bottom microtiter plate and incubated with effectors at the indicated E/T ratios at 37°C for 6–8 h. Percent specific release was calculated using the following formula: [E − S/(M − S)] × 100, where E is the average experimental release of duplicate samples, S is the average spontaneous release of triplicate samples and M is the average maximum release of triplicate samples. Spontaneous release was measured in the absence of effector cells, while maximum release was measured in the presence of 1% NP-40 instead of effector cells.

Preparation of Peptide Extract. Cell suspensions prepared from the spleens of C57BL/6 mice were washed extensively and then homogenized in 0.1% TFA. The suspension was adjusted to pH 2.0 with HCl, sonicated 20 × 1 s and then incubated at 4°C for 60 min. Cellular debris was removed by centrifugation at 2000 × g for 20 min and the supernatant was then passed through a Centrifuge 10 membrane, lyophilized, and resuspended in sterile distilled water.

Production and Purification of Soluble H-2Kb−. Soluble H-2Kb−was prepared as described (35). In brief, a truncated form of H-2Kb−encoding a six-histidine tail after amino acid 273 of the mature protein was inserted into the Drosophila expression vector pR M Ha-3. A full-length CDNA encoding mouse β2 microglobulin was likewise introduced into pR M Ha-3. These constructs were co-transfected together with pHSneo into Drosophila melanogaster SC2 cells and cells were selected in geneticin-containing medium. Protein expression was induced by the addition of CuSO4 and the cell supernatant was harvested after 3 d. The concentrated cell supernatant was purified on an Ni-NTP column followed by ion exchange chromatography.

A say for Stimulation of H hybridoma by Immobilized H-2Kb−. Immlun 3 plates were coated with 50 μl/well of soluble H-2Kb−at a concentration of 5 μg/ml for 2 h at 37°C and then washed twice with PBS. Hybridoma T cells were added to the coated plate at a concentration of 107 cells and incubated overnight at 37°C. β-galactosidase activity was assayed using 5 mM ONPG (o-nitrophenol-β-galactopyranoside) as the colorimetric reagent (33). Absorption was read in a ELISA plate reader at 405 nm 4 h after addition of the developing reagent. The immobilized H-2Kb−was acid treated to remove any bound peptide by adding 100 μl of 100 μM sodium acetate/acetic acid buffer (pH 3.0) to H-2Kb−-coated
Anti-H-2K\(^b\)-alloreactive CTL Clones Can Kill T2 Cells Expressing H-2K\(^b\). Allo H-2K\(^b\) reactive CTL clones were isolated from the H-2\(^d\) mice, (BALB/c \(\times\) B10.D2)F\(_1\), after stimulation with cells from strain 29.7. Strain 29.7 are B10.D2 (H-2\(^b\)) mice transgenic for an H-2K\(^b\) gene that carries a substitution in the \(\alpha_3\) domain (glutamic acid to lysine at residue 227) which prevents association with CD8. The F\(_1\) mice were primed in vivo with a skin graft and, after rejection of the graft, CTL clones were isolated from the spleen by in vitro restimulation. A total of 88 clones were isolated. Of these 88 clones, 80 were able to kill an H-2K\(^b\) transfectant of the antigen-processing-defective cell line T2, with a specific lysis of 22% or greater (Fig. 1). From this panel of CTL clones, 6 were selected for further analysis.

The initial indication that killing by these six clones was independent of peptide was their ability to lyse T2 cells transfected with the H-2K\(^b\) gene (Fig. 2). The killing was H-2K\(^b\) specific because there was no reactivity for non-transfected T2 cells (Fig. 2) nor T2 cells transfected with H-2L\(^d\) (data not shown). All six clones were also tested for their ability to kill M12.C3 (H-2\(^a\)) as well as L cells (H-2\(^b\)) and in all cases lysis was only observed with transfected target cells that express H-2K\(^b\) (data not shown). Further demonstrating peptide independence, the addition of an extract containing peptides recovered from C57BL/6 (H-2\(^b\)) spleen cells did not enhance the killing of T2.K\(_b\) by these 6 clones (Fig. 2). This extract was capable of sensitizing T2.K\(_b\) cells for lysis by the H-2K\(^b\) specific, peptide-dependent alloreactive T cell clone 4.1 (Fig. 2). We also compared the reactivity of our six peptide-independent clones (three of which are shown in Fig. 3) for wild-type T1.K\(_b\) and mutant T2.K\(_b\) cells. Also
shown is their reactivity for T2.Ld cells. The peptide-independent CTL were able to lyse both Kb transfected targets to a similar degree, whereas the 4.1 clone killed T1.Kb but not T2.Kb cells. Furthermore, treatment of T2.Kb cells with acid to remove peptides bound to MHC molecules did not abolish the reactivity of the CTL for T2.Kb (Fig. 4). To demonstrate the effectiveness of acid stripping, we showed (Fig. 4) that it abolishes killing, by the peptide-dependent clone 4.1, of T2.Kb targets pulsed with C57BL/6 spleen cell extract. Although the reactivity of the peptide-independent clones for T2.Kb was somewhat decreased after acid treatment (Fig. 2 versus Fig. 4), this is probably a consequence of target cell damage inflicted by the acid. Such treatment kills between 30 and 50% of the targets, and the E/T ratio reflects targets that are still viable after acid stripping. This experiment was also performed using CTL clones 20, 40, and 59, along with the peptide-dependent control, 4.1, on M12.Kb target cells. As shown in Fig. 5, the acid treatment completely abolished the reactivity of 4.1 for M12.Kb cells, but only partially inhibited reactivity of clones 20 and 40 for M12.Kb. The lytic capacity of clone 59 for M12.Kb was not affected by the acid treatment. Lysis of the M12 targets by these CTL demonstrates specificity for H-2Kb as M12.Kb, but not M12.C3 cells, were killed.

The anti-H-2Kb-alloreactive CTL can kill RMA-S cells. Although the T2 cell line does not express serologically detectable levels of HLA molecules on its surface, transfection of mouse class I genes, such as H-2Kb, leads to very high levels of serologically detectable product on the surface of the T2 cells. We examined whether our CTL clones would recognize H-2Kb on the surface of the H-2Kb cell line RMA-S, which also is defective in antigen processing and, when cultured at 37°C, does not express any serologically detectable H-2Kb. Despite this low level of expression of H-2Kb on RMA/S, they were readily killed by the CTL clones (Fig. 6). The ability of the CTL clones to recognize H-2Kb on the surface of T2 and RMA-S cells also suggests that these CTL do not require the presence of a particular peptide derived from a particular cytosolic protein.

Peptides present in fetal calf serum do not contribute to the determinant recognized by CTL. A possible interpretation of the above observations is that there are peptides in the FCS in the culture media that are capable of binding to H-2Kb on the surface of T2.Kb and RMA-S cells and that the CTL, which were isolated in vitro, recognized a determinant comprised of this peptide. This possibility was examined by culturing the target cells in serum-free media for 10 d before use in a cytotoxicity assay which was also performed in serum-free conditions. As shown in Fig. 7, target cells cultured in serum-free media were readily lysed by all six of the CTL clones tested, demonstrating that recognition by these T cells do not require FCS-derived peptides.

The anti-H-2Kb CTL clones are also capable of killing T2 cells expressing H-2Kbm8. The H-2Kbm8 molecule has mutations at residues 22, 23, 24, and 30, which lead to differences from wild-type H-2Kb in the peptides bound and presented (36, 37), as well as changes in the conformation of bound peptides common to both H-2Kb and H-2Kbm8.
The ability of all of our anti H-2Kb CTL clones to kill T2 cells expressing H-2Kbm8 molecules (Fig. 8) is consistent with these T cells recognizing determinants that are not dependent upon particular peptides.

Recognition of H-2Kb Produced in a Drosophila Expression System, by Alloreactive T Cells. An expression system using Drosophila melanogaster cell lines has been used to produce quantities of soluble H-2Kb molecule (39). The H-2Kb isolated from this system are mostly devoid of peptides (39, 40), although some of the H-2Kb molecules contain a peptide derived from the yeast extract used as a supplement in the culture media. This yeast-derived peptide has low affinity for H-2Kb and does not possess the motif found in peptides which bind to H-2Kb with high affinity. To assay the ability of the anti-H-2Kb alloreactive T cells to recognize the H-2Kb produced by Drosophila melanogaster cells, T cell hybridomas were produced. The fusion partner used was a derivative of the BW5147 line transfected with a β-galactosidase reporter gene inserted behind the IL-2 promoter (33). Upon engagement of the TCR, hybridomas produced using this fusion partner produce β-galactosidase which can be detected by a colorimetric assay. Hybridomas were prepared from clones 34 and 59, and both reacted readily with T2 cells transfected with H-2Kb (data for hybridoma 34.9, derived from clone no. 34, shown in Fig. 9A) and not with T2 cells transfected with the H-2Ld molecule (data not shown).

Both of the hybridomas reacted strongly with H-2Kb immobilized on the surface of an ELISA plate (data for hybridoma 34.9 shown in Fig 9B). Therefore, the T cells were able to recognize H-2Kb produced by the Drosophila expression system. To confirm that this reactivity was not dependent upon a peptide associated with the H-2Kb produced by the Drosophila cells, the immobilized H-2Kb was treated with sodium acetate/acetic acid buffer (pH 3.0) to remove any bound peptide, before addition of the hybridoma cells. Serological analysis demonstrated that the acid treatment did not remove a significant amount of the bound H-2Kb (data not shown). The reactivity of the alloreactive hybridomas was unaffected by the acid treatment of the immobilized H-2Kb (Fig. 9B). The efficiency of the acid treatment was demonstrated by its ability to completely remove the ovalbumin-derived peptide SIINFEKL, which binds to H-2Kb with high affinity. The reactivity of a hybridoma derived from the H-2Kb-SIINFEKL-reactive clone, B3 (B3.53), for peptide pulsed H-2Kb was abolished after acid treatment (Fig. 10). Overall, this data demonstrates that the anti-H-2Kb T cell hybridomas recognize the H-2Kb produced by Drosophila cells in a peptide-independent manner.

Peptide-independent Alloreactive CTL Are Enriched after a Skin Graft. The precursor frequency of CTL reactive
with T2.Kb and M12.Kb cells was determined in naive mice and mice primed in vivo with an H-2Kb-incompatible skin graft. The skin graft donors were transgenic mice expressing either the wild-type or the mutant (CD8 non-binding) H-2Kb gene. The frequency of H-2Kb-alloreactive T cells that reacted with either M12.Kb or T2.Kb cells after primary in vitro stimulation with the wild-type H-2Kb molecule was greater than the frequency observed after stimulation with the mutant H-2Kb molecule (Table 1). For either in vitro stimulus, the pCTL frequency of T cells reactive to M12.Kb targets was greater than the frequency of T cells reactive to T2.Kb targets. In mice grafted with either mutant or wild-type H-2Kb incompatible skin, the pCTL frequencies of T cells reactive with T2.Kb targets were comparable to that of CTL reacting with M12.Kb (Table 1). Thus, skin grafting enriches for T2.Kb-reactive CTL.

To determine whether the dramatic increase in peptide-independent CTL after skin grafting was confined to lysis of T2.Kb targets, pCTL assays were also performed using RMA-S targets. The frequency of pCTL reactive with RMA-S targets was similar to the frequency of pCTL reactive to T2.Kb targets. To confirm that the reactivity observed is not a consequence of the recognition of the limited repertoire of peptides expressed by H-2Kb on T2 and RMA-S cells, the pCTL frequency for acid treated T2.Kb targets was determined, and found to be similar.

To determine whether the frequency of pCTL reacting with T2.Kb targets was greater than the frequency observed after stimulation with the mutant H-2Kb molecule (Table 1). For either in vitro stimulus, the pCTL frequency of T cells reactive to M12.Kb targets was greater than the frequency of T cells reactive to T2.Kb targets. In mice grafted with either mutant or wild-type H-2Kb incompatible skin, the pCTL frequencies of T cells reactive with T2.Kb targets were comparable to that of CTL reacting with M12.Kb (Table 1). Thus, skin grafting enriches for T2.Kb-reactive CTL.

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Figure 8. Cytotoxic reactivity of the six H-2Kb-specific alloreactive CTL clones for T2.Kbm8 target cells. The clones are denoted by the following symbols: (filled squares) 20; (filled triangles) 34; (filled circles) 39; (open squares) 40; (open triangles) 41; (open circles) 59.

Figure 9. (A) Reactivity of alloreactive anti H-2Kb hybridoma 34.9 with non-transfected T2 and T2 cells transfected with H-2Kb. (B) Reactivity of hybridoma 34.9 to immobilized H-2Kb pulsed with the SIINFEKL peptide. Activation was determined by a colorimetric assay with ONPG as the β-galactosidase substrate. Absorbance was read at 405 nM. B3.53 is stimulated only by H-2Kb in the presence of SIINFEKL peptide, and acid treatment of this complex removes the peptide and abrogates the ability of the complex to stimulate the hybridoma.

Figure 10. Reactivity of the H-2Kb ovalbumin (SIINFEKL)-specific hybridoma B3.53 to immobilized H-2Kb pulsed with the SIINFEKL peptide. Activation was determined by a colorimetric assay with ONPG as the β-galactosidase substrate. Absorbance was read at 405 nM. B3.53 is stimulated only by H-2Kb in the presence of SIINFEKL peptide, and acid treatment of this complex removes the peptide and abrogates the ability of the complex to stimulate the hybridoma.
A peptide-independent anti-H-2Kb-alloreactive T cell clone is capable of mediating graft rejection. The ability of one of the anti-H-2Kb CTL clones, no. 59, to mediate rejection of an H-2Kb-compatible skin graft was examined in thymectomized, T cell-depleted mice. Neonatally thymectomized CD2F1 mice were depleted of T cells by multiple injections of antibodies to CD4 and CD8. These mice were then grafted with skin from 29.7 transgenic mice. At 60 d after engraftment, 107 clone no. 59 cells were injected intravenously. In 8 out of 8 mice, the grafts were totally rejected within 16 d (range 10–16 d) after injection of the CTL clone. In contrast, mice not injected with clone no. 59 retained the H-2Kb-compatible graft without any sign of rejection at 120 d after grafting. Thus, clone no. 59 is capable of mediating graft rejection.

### Table 1. Frequency of Anti-H-2Kb-alloreactive CD2F1 Spleen Cells after a 7-d MLR with Stimulator Spleen Cells Expressing Either Wild-Type H-2Kb or Mutant H-2Kb That Does Not Engage MHC Class I

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C57BL/6 extract

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*CD2F1 mice were grafted with 28.1 (transgenic for wild-type H-2Kb, denoted Kb.wt) or 29.7 (transgenic for mutant H-2Kb that does not engage CD8, denoted Kb.m) trunk skin. Spleen cells were harvested 14 d after skin grafting. At this time, the grafts were completely rejected.*

MLR stimulators were either 28.1 (Kb.wt) or 29.7 (Kb.m) spleen cells. The MLR represents either a primary anti-H-2Kb in vitro stimulation of cells from naive mice, or a secondary in vitro stimulation of spleen cells from mice that received an H-2Kb (wild type per mutant)-incompatible skin graft.

with T2.Kb in the presence or absence of the extract. Similarly, the pCTL frequency for reactivity with T1.Kb, the counterpart of T2.Kb that processes peptide normally, was similar to the pCTL frequency for T2.Kb. Thus, skin graft priming enriches for peptide-independent alloreactive CTL.

The reactivity of the majority of alloreactive T cells is considered to be peptide specific. This conclusion is based on several studies in which most of the alloreactive T cells characterized did not recognize class I molecules on T2 or RMA-S cells without the addition of peptide extracts prepared from normal cells expressing the relevant MHC class I (5–8). When lysis of peptide-processing-defective targets was observed, it was usually enhanced by additions of exogenous peptide extracts from wild-type cells, suggesting that the determinant recognized was actually peptide dependent (6, 13, 15). In some instances, including the anti-H-2Ld allo clone 2C (9), as well as clones alloreactive with Qa-1 (41), H-2Kb (42), and HLA-A3 (43) the sequence of the allopeptide has been determined. In addition, stimulation with allogeneic APCs pulsed with a specific peptide can elicit peptide-specific alloreactive T cells (10). Further evidence for peptide specificity of alloreactive T cells was provided by experiments that demonstrated that some T cell clones require peptides that are specific for either certain tissues (7, 11) or species (12). In contrast, the data presented herein demonstrates that some anti-H-2Kb alloreactive CTL clones are able to recognize determinants that do not require a particular peptide. We established this by several criteria: (a) the ability to recognize H-2Kb on the antigen presentation-defective cell lines T2 and RMA-S, (b) the inability of a peptide extract eluted from C57BL/6 spleen cells to enhance the killing of these antigen processing-defective target cells; (c) the failure of acid treatment of target cells to reduce CTL killing; (d) recognition of the mutant molecule H-2Kbm8; (e) recognition of plate-bound H-2Kb even after treatment with acid to remove any bound peptide. The identification of these cells confirms that, in addition to peptide determined ligands, the polymorphic differences of the MHC molecules themselves can define allo determinants for T cell recognition.

Although it has been established that many alloreactive T cells are peptide specific, such evidence does not exclude the possibility of peptide-independent allorecognition by some T cells. Peptide-independent allorecognition was first proposed by Bevan (4) who suggested that it was influenced by high determinant density on the target cell. According to this model MHC determinants that are not influenced by peptide are present on most of the target molecules thereby enabling a high density ligand to compensate for the low affinity of the TCR for the alloantigenic molecule (44, 45). Indeed, the neonatal T cell repertoire exhibits peptide promiscuity in T cell recognition (46) suggesting that these T cells recognize the MHC molecule directly. Moreover, there are several reports characterizing alloreactive CTL that are not peptide specific (12, 13, 15, 16, 47). In addition it has been shown that some alloreactive γδ T cells are able to recognize determinants unaffected by mutations in the MHC molecule that alter peptide presentation of self-MHC-restricted αβ T cells (48).

Perhaps the most definitive evidence for peptide-independent allorecognition is provided by a CTL line that...
proliferated upon incubation with plate-bound HLA-A2 that had been denatured, purified, and renatured in the absence of peptide (16). The authors did not, however, establish the validity of the system by incorporating a peptide-dependent control, nor did they address the possibility that a peptide in the FCS bound to the isolated class I molecule and contributed to the alldeterminant. Several other reports have also described CTL clones that may recognize peptide-independent ligands. For example, alloreactive T cell clones which can recognize H-2Kb on RMA-S (13, 15), or either H LA-A2 (14, 16) or transfected murine class I molecules on T2 (12) cells have been described. Some of these alloreactive CTL lyse RMA/S to the same extent as they kill RMA, the wild-type counterpart of RMA/S (13). In such studies the possibility that class I molecules on RMA/S and T2 can present peptides for which such CTL cells are specific was not directly addressed. Attempts by others to deal with this issue have involved extracting peptides from RMA/S cells (15) in order to isolate particular fractions to add back to RMA/S cells for higher density presentation. Although lysis by some alloreactive clones was enhanced by pulsing RMA/S targets with a particular peptide fraction, the recognition by other clones appeared to be truly peptide independent. The interpretation of all these observations, then, is complicated by evidence that the T2 and RMA/S cell lines can present a repertoire of peptides including those derived from either signal sequences in the endoplasmic reticulum (17, 18), from minigenes that incorporate sequences for targeting fusion proteins to the endoplasmic reticulum (19) or from influenza (21), Sendai (20), murine leukemia (22), and vesicular stomatitis (21, 23) viruses. Overall, such observations demonstrate that the inability of RMA-S and T2 cells to present peptides in association with class I molecules is not absolute. Therefore, killing these targets by alloreactive CTL is insufficient evidence to conclude that such CTL recognize peptide-independent epitopes. For such reasons the existence of peptide-independent alloreactive T cells is controversial, and for the most part, widely overlooked.

Among the alloreactive CTL that are able to kill RMA/S or T2 cells, there are three discernible phenotypes distinguished by alterations in their reactivity resulting from the addition of exogenous peptides capable of binding to the target MHC class I molecule (6, 15). The reactivity of many alloreactive CTL that are able to kill RMA/S or T2 is enhanced by the addition of peptides eluted from wild-type cells expressing the target molecule, suggesting that such CTL are peptide dependent. A second phenotype is manifest by CTL that can kill RMA/S or T2 cells in the absence of added peptide, but if the targets are pulsed with high concentrations of a single synthetic peptide able to bind the molecule for which they are specific, the reactivity is decreased (13) or abolished (Smith, P.A., and T.A. Potter, unpublished observations). Such T cells are considered to recognize either empty class I molecules, class I molecules containing one of several peptides that result in a particular conformation, or class I molecules containing a specific peptide that is displaced by the addition of the exogenous synthetic peptide. A third phenotype is comprised of CTL that lyse RMA/S even in the presence of high concentrations of added synthetic peptide (13). Such CTL could be reacting either with a peptide that is not displaced by the added peptide or, alternatively, these CTL may recognize a determinant that is unaffected by the presence or absence of a particular peptide. We have determined (data not shown), that pulsing T2.Kb target cells with high concentrations of synthetic peptides such as SIINFEKL, did not displace peptides already bound to H-2Kb. For example, the peptide-dependent 4.1 CTL clone lysed T2Kb cells pulsed with peptide extracted from C57BL/6 spleen cells. This reactivity was ablated by treatment of the target cells with acid to remove peptides bound to MHC molecules, but it was not diminished by the addition of micromolar amounts of SIINFEKL. Thus, pulsing with high concentrations of synthetic peptide, unlike acid treatment, does not necessarily displace all MHC-bound peptides.

The killing of RMA/S or T2.Kb cells by the CTL we describe here was not enhanced by the addition of peptide extract derived from C57BL/6 spleen cells, nor was it diminished by acid treatment of the target cells. To further characterize whether peptides were required for the determinant recognized by our CTL, we examined the reactivity with the H-2Kbm8 molecule which carries several substitutions that lead to differences in allelogenicity and peptide binding compared to wild-type H-2Kb (37). In addition to binding their own unique set of peptides, the H-2Kbm8 and H-2Kb molecules also bind some common peptides, although these overlapping peptides are bound in a different conformation by the two molecules (49). The observation that our alloreactive CTL are able to recognize both H-2Kb and H-2Kbm8 is consistent with a peptide independence of the epitope recognized. The CTL clones that we generated were isolated in vitro in the presence of fetal bovine serum, thus it was a formal possibility that the serum contained a peptide which was capable of binding to H-2Kb on the cell surface and thereby eliciting a response specific for this complex. This possibility is particularly relevant when assaying on transfectants of T2 cells, because they express transfected murine MHC class I molecules which bind exogenous peptide very efficiently. Culture of the transfected T2 cells in serum-free conditions did not affect killing by the CTL, indicating that FCS derived peptides did not contribute to the epitope(s) recognized. Finally, we derived hybridomas from two of our CTL clones, so that we could analyze the response to H-2Kb produced in Drosophila melanogaster cells which do not possess the ability to load peptide (39, 40). The hybridomas reacted strongly to the immobilized H-2Kb even after the molecule had been treated with acid to remove any bound peptide. This observation provides very strong evidence that the recognition of H-2Kb by these T cells is peptide independent.

After skin graft priming, there was an increase in the pCTL frequency of CTL reacting with both RMA/S and T2.Kb. To confirm that this reactivity does not represent recognition of the limited repertoire of peptides expressed in association with H-2Kb on these target cells, we assayed
the ability of these CTL to kill acid-treated target cells. The pCTL frequency of T cells recognizing T2.Kb was unaffected either by treatment of the target cells with acid to remove MHC-bound peptides or by addition of an extract containing MHC-bound peptides from cells which express H-2Kb. Furthermore, the frequency of pCTL reacting with T1.Kb, the counterpart of T2.Kb which processes peptide normally, was essentially the same as the pCTL frequency of cells reacting with T2.Kb.

In our efforts to isolate peptide-independent alloreactive cells, we initially used skin graft priming followed by an MLR with stimulator cells expressing a mutant form of H-2Kb that does not engage CD8. Our rationale was that, by selecting for T cells that do not require CD8 engagement for activation, we would isolate either T cells that recognize H-2Kb in association with a particular peptide with very high affinity or, alternatively, recognize a high-density ligand with a lower affinity. The latter possibility is based on previous reports (50–52) which demonstrated a correlation between CD8 dependence and ligand density. Thus, by removing the ability of the TCR and the CD8 molecule to simultaneously engage H-2Kb, T cells that can recognize high-density ligands may be selected. The examples of high-density allo determinants would include those that do not require a particular peptide, either (a) epitopes common to all H-2Kb complexes, (b) epitopes dependent on highly abundant peptides, (c) epitopes shared by H-2Kb associated with several different peptides, and (d) empty H-2Kb. As our precursor frequency data shows, abrogation of CD8 engagement does decrease the reactivity to H-2Kb-expressing targets in a primary MLR by about an order of magnitude, suggesting that, in the absence of CD8 engagement, the frequency of H-2Kb-reactive CTLs in unprimed mice is low. The frequency of pCTL recognizing the mutant H-2Kb molecule is increased dramatically after priming in vivo with a skin graft. Subsequent experiments also revealed that the frequency of pCTL reactive with T2.Kb alloreactive cells is comparable whether or not the H-2Kb expressed by the skin graft and MLR stimulators can engage CD8. Thus, skin grafting is very efficient for enriching and selecting for peptide-independent alloreactive T cells. We have recently isolated an additional 9 CTL lines from mice primed with and restimulated in vitro with wild-type H-2Kb which behave similarly to the clones described in this paper. All of these 9 lines are H-2Kb specific, lyse T2.Kb and T1.Kb cells with the same efficiency, and their lysis of T2.Kb cells is not enhanced by the addition of the C57BL/6 spleen cell extract (data not shown).

Skin grafting is a very potent allo-stimulus, and may be very efficient in selecting T cells that recognize a high-density ligand such as peptide-independent epitopes. Although evidence for peptide-independent alloreactive T cells has previously been reported (12–15), they are generally found at a low frequency relative to peptide-dependent T cells. This may be related to the methodology used for their isolation, particularly in terms of in vivo priming. Many of the characterized alloreactive T cells are obtained from a primary in vitro stimulation or after interperitoneal injections of spleen or tumor cells expressing allo determinants. These conditions may not adequately encompass all of the alloresponse. This is particularly apparent in our studies which demonstrated the enhanced selection of peptide-independent T cells by priming with a skin graft. Skin contains a large number of Langerhans cells that, upon transplantation, migrate to local lymph nodes of the recipient and activate naïve T cells (53, 54). Moreover, a correlation between Langerhans cell density and the rapidity of skin graft rejection has been established (54). Perhaps, then, the fine specificity of alloreactive T cells isolated after potent stimulation by Langerhans cells includes T cells that recognize a ligand present at a high density, namely the H-2Kb molecule regardless of peptide occupancy.

The observation that transfer of a peptide-independent CTL clone was sufficient to confer the ability to reject an H-2Kb incompatible skin graft, suggests that further studies of the fine specificity of alloreactive T cells elicited during the rejection of different tissues are required. This is particularly applicable for prospective clinical treatments to circumvent transplantation rejection. For example, based upon studies showing peptide-restricted allo-responses of CD4 cells to MHC class II molecules (55, 56), it has been suggested that the prevention of allograft rejection might be accomplished by induction of tolerance to specific peptide determinants (56). Since our peptide-independent clones were primed by a graft in which the only disparity was the class I H-2Kb molecule, the success of this suggested approach in preventing rejection of a MHC class I disparate graft would depend on whether the stimulation of these cells in vivo is helper cell dependent, and, if so, whether or not the activation of the helper cells is peptide dependent.

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1031 Smith et al.
References


1032 Peptide-independent R recognition by Alloreactive CTL
45. Janeway, C.A. 1992. The T cell receptor as a multicompo-
43. Poindexter, N.J., B. Naziruddin, D.W. McCourt, and T.
35. Sykulev, Y., A. Brunmark, M. Jackson, R.J. Cohen, P.A.
34. Matsumura, M., Y. Saito, M.R. Jackson, E.S. Song, and P.A.
31. Matsumura, M., Y. Saito, M.R. Jackson, E.S. Song, and P.A.
29. Poindexter, N.J., B. Naziruddin, D.W. McCourt, and T.
27. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
25. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
23. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
22. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
20. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
17. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
15. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
12. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
11. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
10. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
8. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
7. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
5. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
4. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
2. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.
1. Shimonkevitz, R., B. Leuscher, J.-C. Cerottini, and R.H.