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Alloreactive Cytotoxic T Lymphocytes Generated in the Presence of Viral-Derived Peptides Show Exquisite Peptide and MHC Specificity[1]

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Abstract. The nature of alloreactivity to MHC molecules has been enigmatic, primarily because of the observation that allogeneic responses are considerably stronger than syngeneic responses. To better determine the specificity potential of allogeneic responses, we have generated alloreactive CTL specific for exogenous, viral-derived peptide ligands. This approach allowed us to critically evaluate both the peptide- and MHC-specificity of these alloreactive T cells. Exploiting the accessibility of the H-2L^d class I molecule for exogenous peptide ligands, alloreactive CTL were generated that are specific for either murine cytomegalovirus (MCMV) or lymphocytic choriomeningitis virus (LCMV) peptides bound by L^d alloantigens. Peptide specificity was initially observed in bulk cultures of alloreactive CTL only when tested on peptide-sensitized T2.L^d target cells that have defective presentation of endogenous peptides. Subsequent cloning of bulk alloreactive CTL lines generated to MCMV yielded CTL clones that had exquisitely specific MCMV peptide recognition requirement. All of the MCMV/L^d alloreactive CTL clones were also exquisitely MHC-specific in that none of the CTL clones lysed targets expressing MCMV/L^q complexes, even though L^q differs from L^d by only six amino acid residues and L^q also binds the MCMV peptide. This observation clearly demonstrates that alloreactive CTL are capable of the same degree of specificity for target cell recognition as are syngeneic CTL in MHC-restricted responses. *Journal of Immunology,* 1993, 151: 1.

Ag-specific, MHC-restricted CTL recognize a complex of peptide Ag presented in the context of self-MHC molecules; however, it remains unclear whether alloreactive CTL recognize MHC by a similar mechanism. Extensive studies in allorecognition have yielded several mechanistic models: 1) alloreactive CTL recognize only the foreign MHC in the absence of a bound peptide, 2) alloreactive CTL recognize a conformational stabilization or change induced by a bound peptide, thereby requiring the presence of peptide but not interacting directly with it, or 3) alloreactive CTL recognize and interact with specific determinants on both the bound peptide as well as the allogeneic MHC molecule. The last model would suggest that alloreactive CTL recognize alloantigens in a manner analogous to recognition by Ag-specific, self-restricted CTL.

Discrimination among these models has proven difficult. A study by Elliott and Eisen suggests that recognition of allogeneic MHC molecules can occur in the absence of bound peptide in that purified HLA-A2 molecules reconstituted in the absence of peptide could serve as an efficient recognition element for a CD8$^+$ anti-A2 CTL line (1). However, the possibility that contaminating peptides remained at low levels in the purified MHC preparation could not be excluded.

The involvement of peptide in allorecognition has been demonstrated using the peptide transport-defective T2 cell line (2). K^b alloreactive CTL would only lyse T2.K^b target cells if they were pulsed with cyanogen bromide fragments of cytoplasmic proteins. Moreover, HPLC fractionation of the cleavage products revealed that individual clones were specific for different HPLC fractions. Similar peptide de-

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pendencies have been confirmed by a number of other groups (3,4). Finally, a study by Rötzschke et al (5) postulates the existence of alloreactive CTL that can be grouped into three general categories: those that do not require peptide, those that require a specific peptide, and those for which different peptides appear to allow for recognition. Given the complexity of the reported data, it is possible that an alloresponse is the sum of a heterogeneous population of responding CTL that exhibit a spectrum of peptide requirements. However, the extent to which any specific type of alloreactive CTL contributes to the total allogeneic response is unknown.

Previous studies have attempted to identify a role for peptide ligand in the recognition of allogeneic MHC, utilizing alloreactive clones purportedly specific for an unknown endogenous peptide ligand presented by alloantigen. Identification of the peptide ligand specificity of these clones will likely be difficult given the large number of endogenous peptide Ag postulated to be presented by self-MHC molecules. Furthermore, without identification of the peptide specificity of alloreactive CTL, it has been impossible to investigate the separate contribution of MHC vs peptide determinants.

To more specifically address the involvement of peptide and MHC in allorecognition, we have developed an assay system devised to generate alloreactive CTL to known peptide/MHC complexes. Interestingly, early studies by Ishii et al (6) suggested that alloreactive T cells specific for class II MHC molecules could be generated to specific nominal Ag. In the present study CTL were generated to the alloantigen H-2L^d in the presence of either the MCMV[3] (7) or LCMV (8) peptide. L^d is particularly well suited for these analyses because of the accessibility of its peptide binding site. Previous reports from our lab have demonstrated that L^d can readily bind exogenous ligand as assessed by increased cell surface expression after incubation with peptide (9). The observed induction results from the stabilization of L^d molecules by significantly prolonging their surface half-life (10). Direct binding of exogenous peptides has been demonstrated and correlates with the observed induction in cell surface expression.

Allogeneic responses to peptide/L^d complexes were generated by stimulating splenocytes from the L^d loss mutant strain dm2 (K^{d+},D^{d+}, L^{d-}) with BALB/c (K^{d+}, D^{d+}, L^{d+}) splenocytes in the presence of either the MCMV or LCMV peptide. CTL lines generated against MCMV/L^d or LCMV/L^d complexes lysed the class I transport-defective cell line T2.L^d only if pulsed with the appropriate peptide. When MCMV/L^d-specific alloreactive CTL were examined at the clonal level, a stringent requirement for both peptide and MHC was observed. Therefore, we conclude that alloreactive CTL exist that require specific peptide presented by alloantigen, and that these CTL recognize allogeneic MHC in a manner analogous to that by which Ag-specific CTL recognize a foreign peptide presented by self-MHC.

[3] Abbreviations used in this paper: MCMV, murine cytomegalovirus; LCMV, lymphocytic choriomeningitis virus; dm2, L^d-loss mutant mouse strain BALB/c-*H-2*^{dm2}

Materials and Methods

Mice

BALB/c Kh (H-2^d) and BALB/c-H-2^{dm2} (dm2,*L^d* loss mutant) mice were bred in the animal facility of Dr. Donald C. Shreffler, Washington University School of Medicine, St. Louis, Mo. BALB/c-H-2^{dm2} is derived from a BALB/c mouse that has deleted the *L^d* gene (11).

Peptides

The amino acid sequence of the MCMV peptide corresponds to residues 168–176 (YPHFMPTNL) of the MCMV immediate early protein pp89 (7). The amino acid sequence of the LCMV peptide corresponds to residues 119–127 (RPQASGVYM) of the lymphocytic choriomeningitis virus protein (8). The tum$^-$ peptide contains amino acid residues 12–24 (ISTQNHRALDLVA) of the mutant protein P91A$^-$ (exon 4) of the tum$^-$ P815 variant (12). Two identified endogenous L^d-binding peptides were utilized, LSPFPFDL (13) and YPNVNIHNF (14). A β-galactosidase derived peptide contains the amino acid residues TPHPARIGL(sequence from M. Bevan). Peptides were synthesized using Merrifield's solid phase method (15) on a peptide synthesizer (model 431A, Applied Biosystems, Inc, Foster City, CA). Peptides were purified (>90%) by reverse-phase HPLC and subjected to purity assessment techniques as previously described (16).

Cell lines

R1.1-L^d was generated by introducing the *L^d* gene into the R1.1(H-2^k), a C58/J-derived thymoma cell line (ATCC# TIB 42) by electroporation and selection in G418 antibiotic. P815 is a DBA/2 (H-2^d) derived mouse mastocytoma cell line (ATCC# TIB 64). L.L^d, L.L^{d}L95I, L.L^{q}H155Y, K157R, L.L^{q}H155Y, and L.L^q were generated by transfection of recombinant plasmids into DAP-3 L cells (U.H.K., manuscript in preparation). The T2 cell line is a somatic cell hybrid of human B and T lymphoblastoid cell lines that expresses low levels of HLA-A2 and undetectable levels of B5 at the cell surface (17). T2.L^d was generated by electroporation of T2 with the *L^d* gene and was a gift of P. Cresswell (Yale University, New Haven, CT). RMA-S, a C57BL/6 derived thymoma (18,19), was the gift of J. Bluestone (University of Chicago, Chicago, IL). RMA-S.L^d was generated by transfection of the *H-2L^d* cDNA via lipofectin and selection in the presence of 0.6 mg/ml G418. Cells lines were maintained in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with penicillin, streptomycin,

glutamine, HEPES and 10% FCS (Hyclone Laboratories, Logan, UT).

Generation of in vitro primary alloreactive CTL

5×10^6 or 7.5×10^6 responding dm2 splenocytes were co-cultured with 3.5×10^6 irradiated (2,000 rads) BALB/c splenocytes + 10^{-4} M peptide (MCMV or LCMV) in 2 ml of sensitization medium per well of a 24-well plate. On day 5 of culture, effector cells were harvested, counted, and resuspended in RPMI 1640 supplemented with HEPES and 10% new-born bovine serum (Hyclone Laboratories, Logan, UT).

Generation of alloreactive CTL lines and clones

CTL lines were established from primary cultures by restimulation with 5×10^6 irradiated BALB/c splenocytes + 10^{-5} M peptide (MCMV or LCMV) in 2 ml of sensitization medium per well of a 24-well plate. Subsequently, lines were restimulated under the same conditions with the addition of 10 U/ml rIL-2 (Biosource, Camarillo, CA). Subclones were established by plating cells at various concentrations in round-bottomed microtiter plates in replicates of 96. The wells contained 1×10^6 irradiated BALB/c splenocytes +/- peptide in 0.2 ml sensitization medium supplemented with 10 U/ml rIL-2. After two additional in vitro restimulations, cells were tested for activity in a standard ^{51}Cr-release assay. Positive wells were selected for expansion based on the likelihood of clonality according to Poisson statistics (20). T2.L^d cells incubated overnight with 10^{-4} M peptide or P815 cells were used as targets. Selected clones were maintained by weekly restimulation in 24-well plates as described above.

^{51}Cr release assay

10^6 target cells were labeled for 1 h with 150–300 μCi of $Na^{51}CrO_4$ (10–25 uCi/ml, Amersham Corp., Arlington Heights, IL) in 100 μl of RPMI-FCS with or without peptide for 1 h at 37°C in 5% CO_2. In some experiments cells were cultured overnight with peptide before labeling. Cells were then washed and plated in 96-well round-bottomed microtiter plates. Effector cells were added at various concentrations. Plates were spun at 50 *g* for 2 min and incubated at 37°C and 5% CO_2 for 4 h. 100 μl of supernatant was counted in an ISOMEDIC gamma counter (ICN Biomedicals, Huntsville, AL). The mean of triplicate samples was calculated and percent ^{51}Cr release was determined according to the following equation:

$$\text{percent } ^{51}\text{Cr release} = 100 \times \left[\frac{\left(\text{experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}\right)}{\left(\text{maximum } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}\right)} \right]$$

where experimental ^{51}Cr release represents counts from target cells mixed with effector cells, control ^{51}Cr release represents counts from target cells incubated in medium alone (spontaneous release), and maximum ^{51}Cr release represents counts from target cells exposed to 5% Triton X-100. For the data presented in this paper, the SEM percent specific lysis was <5% of the value of the mean.

Generation of MCMV/RMA-S.L^d alloreactive primary CTL

7.5×10^6 C57BL/6 splenocytes were cultured in the presence of 3.5×10^5 RMA-S.L^d cells (7,500 rad) in 2 ml of sensitization medium containing 10^{-4} M MCMV peptide per well of a 24-well plate. RMA-S.L^d cells had previously been cultured for 18 h at 25°C in the presence of 10^{-4} M MCMV peptide. On day 5 of culture, effector cells were harvested, counted, and resuspended in RPMI 1640 supplemented with HEPES and 10% new-born bovine calf serum.

Results

Peptide/L^d-specific alloreactive CTL can be readily generated in the presence of exogenous peptide ligand

In previous work from our lab, we demonstrated that L^d surface expression is induced 3- to 4-fold after incubation with an L^d peptide ligand (9). The induced molecules presumably have bound the exogenous peptide, and therefore a high proportion of L^d molecules contain the pulsed peptide. The observed induction results from the stabilization of L^d molecules by significantly prolonging their surface half-life (10). Consistent with this notion, we are able to readily generate peptide-specific syngeneic CTL restricted by L^d (21). We postulated, therefore, that peptide-pulsed L^d molecules may also provide an efficient system for generating peptide-specific alloreactive CTL.

To this end, primary alloreactive CTL were generated in the L^d loss mutant mouse strain dm2. Dm2 lymphocytes were stimulated with irradiated BALB/c(H-2^d) splenocytes in the presence of either the MCMV or LCMV peptide during a 5-day in vitro culture. To enrich for peptide-specific alloreactive CTL, primary cultures were restimulated with H-2^d splenocytes and peptide for 2 additional wk. To address the existence of peptide-specific L^d alloreactive CTL in the lines, we utilized a target cell with increased sensitivity for identifying MCMV/L^d or LCMV/L^d-specific CTL. T2.L^d is a peptide transport-defective cell line that has been transfected with the *L^d* gene (17). Few class I molecules are expressed on the surface of these cells and those that are appear to be either devoid of peptide or to contain low affinity peptides (22). In addition, incubation with an L^d peptide ligand results in a significant increase in L^d cell surface molecules, all of which have presumably bound the

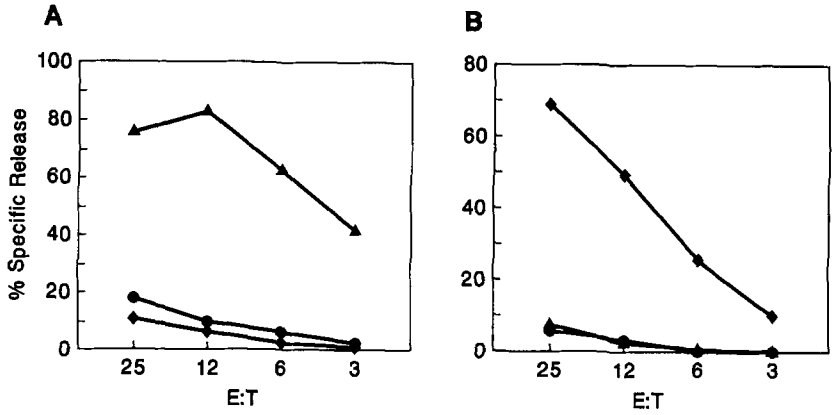


FIGURE 1. L^d alloreactive lines generated in the presence of the MCMV or LCMV peptide are peptide-specific. Responding dm2 splenocytes were co-cultured with irradiated BALB/c stimulators in the presence of 10^{-4} M MCMV or 10^{-4} M LCMV peptide. Cultures were restimulated with BALB/c stimulators and 10^{-5} M peptide for 2 additional wk. At that time effector cells were harvested and cytotoxic activity assessed. Lines stimulated in the presence of MCMV(*A*) or LCMV(*B*) were tested for the ability to lyse T2.L^d(●), T2.L^d+10^{-4} M MCMV overnight(▲), or T2.L^d+10^{-4} M LCMV overnight(◆).

exogenous peptide. Thus, the relative absence of endogenous peptide/L^d complexes should result in a more homogeneous population of MCMV or LCMV peptide/L^d complexes compared with other peptide-induced target cells. In addition, because T2 is a human cell line, it is less likely that endogenous peptides presented by L^d on these cells would be recognized by alloreactive CTL generated by stimulation with murine splenocytes. Together, these attributes should afford increased sensitivity for these target cells in identifying peptide-specific alloreactive CTL in the lines generated in the presence of peptide.

Indeed, the peptide/L^d alloreactive lines demonstrated exquisite specificity in their lysis of peptide-induced T2.L^d cells. Figure 1 shows that CTL stimulated with MCMV/L^d lysed T2.L^d cells induced with the MCMV peptide, but did not lyse T2.L^d incubated with the LCMV peptide even though this peptide induced equivalent levels of L^d surface expression (data not shown). Similarly, CTL stimulated with LCMV/L^d specifically lysed only LCMV-induced T2.L^d target cells (Fig. 1). Therefore, it is evident that peptide-specific alloreactive CTL can readily be generated via stimulation in the presence of L^d peptide ligand. It is noteworthy, however, that these lines recognized R1.1-L^d (H-2^d) target cells very efficiently in the absence of peptide pulsing. In addition, no increase in the lysis of R1.1-L^d cells optimally induced with the generating peptide was observed (Fig. 2). Preferential lysis of peptide-induced target cells may have been expected as a result of recognition by the peptide-specific component within the alloreactive CTL lines. The requirement of the alloreactive lines for the appropriate peptide for T2.L^d lysis, together with the observation that lysis of R1.1-L^d cells is independent of peptide pulsing, suggested that either 1) separate subpopulations existed within the lines, those that recognized endogenous peptides presented by L^d and those specific for the generating peptide presented by L^d; or 2) a cross-reaction existed between the MCMV or LCMV peptide and endogenous peptides presented by R1.1-L^d. To distinguish between these two possibilities, clones were generated from the MCMV/L^d CTL line and evaluated for their peptide requirements.

Alloreactive MCMV/L^d CTL clones demonstrate exquisite peptide specificity

To investigate the existence of two subpopulations within the lines, CTL were cloned from the MCMV/L^d alloreactive line. Initial clonings were carried out in the presence of MCMV peptide and MCMV-specific alloreactive CTL clones were identified based on their ability to lyse MCMV-pulsed T2.L^d target cells. In an independent cloning, CTL were cloned in the absence of MCMV peptide and clones were chosen based on positive lysis of P815 (H-2^d) cells. The latter cloning was designed to specifically identify clones capable of recognizing L^d independent of the MCMV peptide. CTL clones of two distinct specificities were obtained: those that required the MCMV peptide for L^d recognition and those that recognized L^d in the absence of the MCMV peptide.

Clones obtained as a result of stimulation with L^d in the absence of MCMV peptide efficiently lysed non-MCMV pulsed P815 target cells. It is likely that these clones account for the MCMV-independent lysis observed in the alloreactive CTL lines generated in the presence of peptide. Although we have not directly investigated the peptide requirement of these clones, we feel that they are likely to require the presentation of an endogenous peptide based on their inability to lyse T2.L^d cells. This observation suggests a requirement for the presence of a murine-specific peptide present on P815 but missing on T2.L^d.

From two clonings carried out in the presence of MCMV, seventeen clones were obtained. The MCMV specificity of

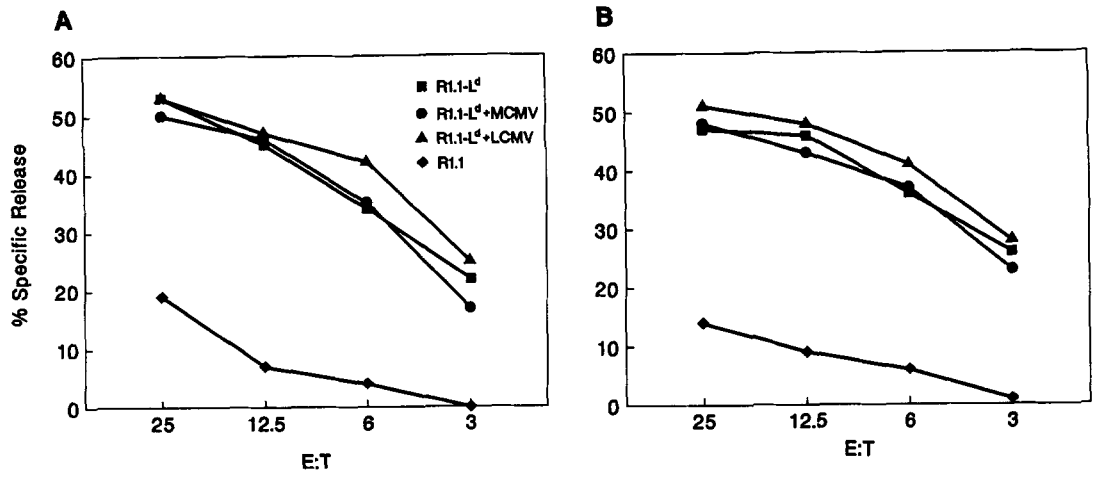


FIGURE 2. Alloreactive CTL lines generated to peptide/L^d complexes do not preferentially recognize R1.1-L^d targets induced with the stimulating peptide. Alloreactive lines generated as described in Figure 1 were tested for the ability to discriminate peptide presented by R1.1-L^d. Lines generated in the presence of (*A*)MCMV or (*B*)LCMV peptide were examined for lysis of untreated R1.1 or R1.1-L^d cells or R1.1-L^d cells induced overnight with 10^{-4} M MCMV or 10^{-4} M LCMV peptide before the assay.

the clones was established by demonstrating the ability to discriminate among a variety of L^d peptide ligands, including viral, tumor-specific, bacterial, and recently defined endogenous peptides. P815 cells pulsed with MCMV, LCMV, tum$^-$, β-gal, ENDO I, or ENDO II (*Materials and Methods*) were used as targets for the alloreactive MCMV/L^d CTL clones. All MCMV/L^d clones analyzed displayed an absolute requirement for the MCMV peptide. Two representative clones are shown in Figure 3. All peptides utilized in this analysis induce equivalent levels of L^d surface expression (23 and data not shown); thus the ability of clones to distinguish among the peptides could not result from differences in the number of allogeneic L^d determinants, but would reflect a specific peptide requirement. In addition, the inability of these clones to recognize P815 (Fig. 3) or R1.1-L^d cells (Fig. 4) in the absence of MCMV peptide suggests that they cannot recognize any endogenous peptide presented by L^d. It is noteworthy that the MCMV/L^d clones were capable of efficiently lysing targets pulsed for 1 h with 10^{-8}-10^{-10} M peptide. These determinant density requirements are similar to those observed for Ag-specific, self-restricted CTL (21) and imply that peptide-specific alloreactive and MHC-restricted CTL are not inherently different in their peptide recognition requirements.

Alloreactive MCMV/L^d CTL clones demonstrate exquisite MHC specificity

The necessity for presentation of the MCMV peptide by L^d was examined by evaluating the ability of the MCMV/L^d alloreactive CTL clones to recognize MCMV presented by the H-2L^q molecule. The L^d and L^q class I molecules differ at only six amino acid positions: 95, 97, 107, 116, 155, and 157 (24). Both molecules bind MCMV to a significant degree (9) and present this peptide physiologically after

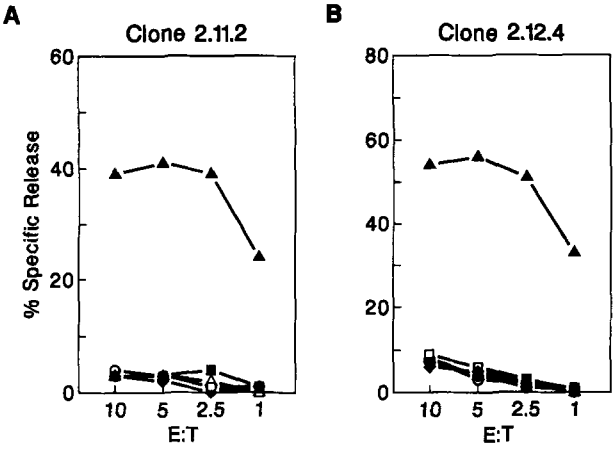


FIGURE 3. Alloreactive MCMV/L^d-specific clones demonstrate an absolute dependence on the presence of the MCMV peptide. Two representative clones are shown. Target cells are P815(●) or P815 pulsed for 1 h with a variety of peptide ligands; P815+MCMV(▲), P815+LCMV(◆), P815+tum$^-$(■), P815+β-gal(□), P815+ENDO I(△), P815+ENDO II(○).

MCMV infection (25). In addition, the R1.1-L^d and R1.1-L^q transfected cells used as targets in this assay express very high and comparable levels of their respective L molecules (data not shown). All 17 alloreactive MCMV/L^d CTL clones examined demonstrated an absolute requirement for MCMV presentation by the L^d molecule, exhibiting no cross-reactive lysis on MCMV-pulsed L^q target cells (Fig. 4). This discrimination between L^d and L^q implies that, in addition to peptide, these MCMV/L^d alloreactive CTL require either direct or indirect recognition of MHC determinants. To investigate the contribution of individual MHC residues in recognition by the MCMV/L^d alloreactive clones, we utilized mutant molecules representing intermediates between L^d and L^q. Mutants chosen for study pos-

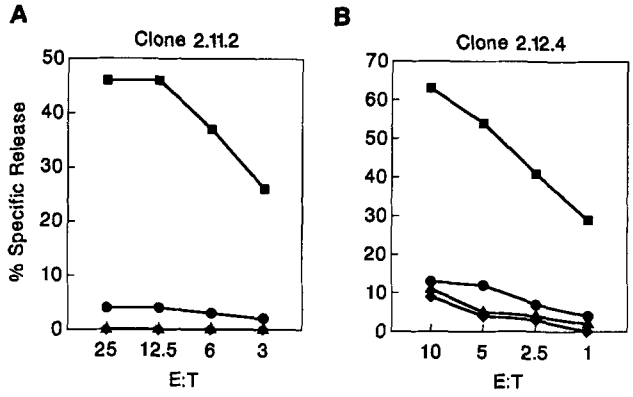


FIGURE 4. Alloreactive MCMV/L^d-specific clones recognize MCMV presented by H-2L^d but not by H-2L^q. The MHC specificity of the MCMV/L^d clones was demonstrated by their ability to discriminate presentation by L^d versus L^q. The cytolytic activity of two representative clones is shown. Targets were pulsed for 1 h with 10^{-4} M MCMV peptide. R1.1-L^d+MCMV(■), R1.1-L^d(●), R1.1-L^q+MCMV(▲), R1.1-L^q(◆).

sessed substitutions at position 155 (L^qH155Y), 155 and 157 (L^qH155Y,K157R), or 95 (L^dL95I). Importantly, parental and variant L^d/L^q molecules were expressed at high levels, and each retained the ability to bind the MCMV peptide as determined by a two-fold or greater induction of L^d cell surface expression by FACS analysis after incubation with peptide (data not shown). Mutants with substitutions at positions 155 and 157 were selected because 155 and 157 are the only two positions that differ within the alpha helical regions of L^d and L^q. It is these regions of the MHC molecule that are postulated to interact directly with the TCR (26) and therefore are likely to be critical for recognition.

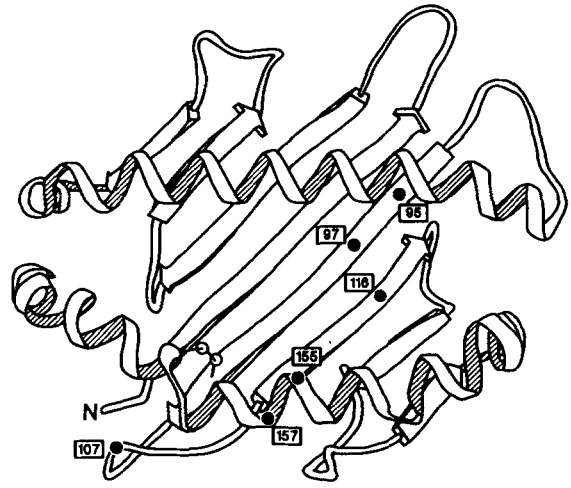
As previously demonstrated, the clones do not recognize MCMV presented by L^q; however, substitutions of the amino acid residues at positions 155 and 157 in L^q (L.L^qH155Y,K157R) with those found in L^d rendered this mutant molecule susceptible to lysis after pulsing with the MCMV peptide (Fig. 5). Interestingly, mutant L.L^qH155Y was recognized by clones as efficiently as L.L^qH155Y, K157R (Fig. 5). Thus position 155 appears to be critical for recognition of L^d by these clones. The involvement of residues at position 155 in allorecognition has been identified by others (27–29). Hence, this position may be commonly used in recognition of MHC by alloreactive CTL. Although MCMV/L^d CTL clones could recognize both L.L^qH155Y,R157K and L.L^qH155Y, recognition of these mutant molecules was less efficient than recognition of the wild type. Even the use of targets maximally induced with peptide before the assay and the presence of continuous peptide during the assay did not allow for recognition at levels equivalent to wild type (data not shown). Therefore interaction of the TCR with residues in the α helices is not, by itself, sufficient for maximal recognition. Consequently, we investigated the contribution of residues within the β-pleated sheet of the MHC molecule.

Residues within the β-pleated sheet of the MHC molecule are suggested to be involved in ligand binding (26). When tested, mutant L.L^dL95I was recognized by the alloreactive MCMV/L^d CTL clones, although at a reduced level compared with the wild type L^d molecule (Fig. 5). When assayed by FACS analysis, these two molecules have similar basal levels of L^d expression and can be induced 3-to 5-fold after incubation with the MCMV peptide (data not shown), thus demonstrating that both molecules are capable of efficiently binding the MCMV peptide. The failure of the helical substitutions to fully restore recognition, and the suboptimal recognition of L.L^dL95I, is consistent with the notion that residues within the β-pleated sheet position the peptide. Consequently, it is possible that differences in peptide positioning could influence CTL recognition. Maryanski et al and Hunt et al (29, 30) have demonstrated the contribution of residues within the β-pleated sheet on recognition by alloreactive CTL. However, the influence of these residues on the bound peptides could not be monitored in these systems. In any case the fact that the MCMV/L^d alloreactive CTL were generated to a known peptide permitted us the unique opportunity to evaluate their MHC specificity apart from their peptide requirements. Furthermore, the demonstration that these alloreactive CTL have a precise peptide and MHC specificity suggests that allorecognition is mechanistically similar to self-restricted recognition of foreign peptide Ag.

Primary alloreactive MCMV/L^d CTL can be efficiently generated when stimulated with MCMV-induced RMA-S.L^d cells

Because the peptide transport mutant T2.L^d permitted peptide discrimination at the target cell level, we postulated that it may also serve as a proficient stimulator for MCMV-specific alloreactive CTL in a primary response. However, our attempts to stimulate a primary response with the T2.L^d cell line were ineffective. Alternatively, we transfected L^d into the RMA-S cell line. RMA-S, like T2, is a transport-defective cell line and is advantageous for this study because of the decreased level of endogenous peptide presentation. In addition, it is a murine cell line and therefore should possess the appropriate accessory molecules that may be required for efficient stimulation of naive T cells.

C57BL/6 (H-2^b) splenocytes were stimulated with RMA-S.L^d cells that had previously been incubated with 10^{-4} M MCMV for 18 h at 25°C. After the 5-day culture period, responder cells were assayed for their ability to discriminate presentation of the MCMV peptide. Figure 6 shows that alloreactive MCMV/L^d-specific CTL can be detected in a primary bulk culture response as demonstrated by the preferential lysis of RMA-S.L^d target cells induced with the MCMV peptide. In all experiments primary CTL generated in the presence of MCMV lysed MCMV-induced



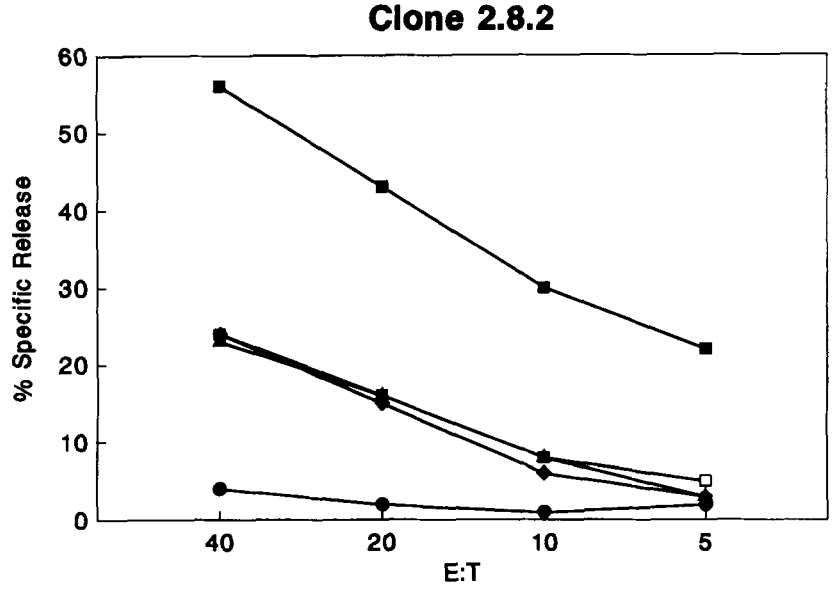


FIGURE 5. Recognition of L^d and L^q mutants by MCMV/L^d-specific clones. *Top*: View of the antigenic groove of L^d. The location of amino acid positions that differ between L^d and L^q are indicated. Differences are given as residue position:L^d amino acid:L^q amino acid-*95*:L:I, *97*:W:R, *107*:G:W, *116*:F:Y, *155*:Y:H, *157*:R:K. *Bottom*: A panel of mutants expressing intermediates between L^d and L^q was used to assess residues important for recognition by the clones. Targets were pulsed for 1 h with 10^{-4} M MCMV peptide: L.L^d(■); L.L^dL95I(□); L.L^qH155Y,K157R(◆); L.L^qH155Y(▲); and L.L^q(●). Mutant and wild type molecules were expressed at high levels on the cells surface. In addition, all targets were capable of binding the MCMV peptide as demonstrated by induction after incubation with MCMV peptide.

RMA-S.L^d at levels significantly above noninduced RMA-S.L^d cells. Thus using cells such as RMA-S as stimulators may be the most direct way to generate alloreactive CTL populations with specificity for a given peptide.

In addition to the ability to detect MCMV/L^d-specific CTL in RMA-S.L^d-stimulated primary responses, initial studies show that MCMV/L^d-specific alloreactive CTL precursors can be detected under conditions of limiting dilution (data not shown). Stimulation with BALB/c splenocytes in the presence of MCMV peptide was sufficient to elicit the MCMV/L^d-specific precursors. Their detection in limiting dilution analyses as well as in primary responses (Fig. 6) suggests that MCMV/L^d-specific alloreactive CTL are not rare clones, but rather represent a readily detectable component of the repertoire of alloreactive CTL.

Discussion

This study clearly demonstrates the existence of peptide-specific alloreactive CTL. It is the first example of the generation of alloreactive CTL to a known peptide/allogeneic MHC complex. This allowed us the unique opportunity to address the role of defined peptides in the recognition of allogeneic MHC, as well as to discriminate between peptide-dependent versus peptide-specific recognition by these alloreactive CTL. Other studies, although demonstrating a role for peptide Ag, have relied on the use of alloreactive clones presumably generated to endogenous peptide/class I complexes (2–5, 31, 32). The involvement of peptide ligand has been demonstrated via addition of cyanogen bromide-derived peptides of cytoplasmic proteins (2), or by blocking recognition through the addition of exogenous peptides (31, 32). These studies, although demonstrating a dependence on peptide ligand, do not address whether these CTL require a specific peptide or whether any peptide (or group of peptides) will suffice. Knowledge of the peptide ligand for which our alloreactive CTL are specific allows us not only to demonstrate peptide involvement in recognition, but to establish the monospecificity of

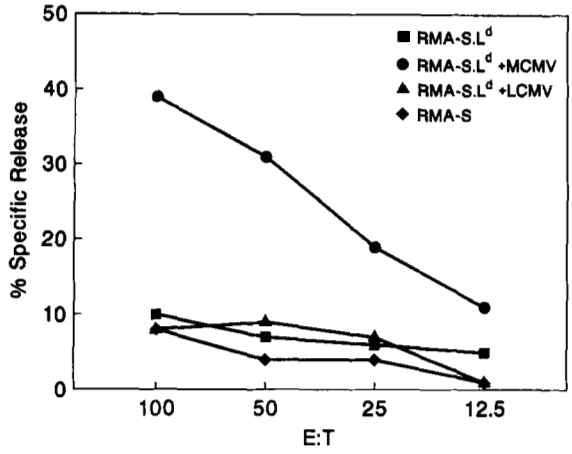


FIGURE 6. MCMV/L^d-specific CTL are readily detectable in an alloreactive primary bulk culture. C57BL/6 splenocytes were co-cultured in the continuous presence of 10^{-4} M MCMV peptide with RMA-S.L^d cells previously induced with 10^{-4} M MCMV at 25°C. On day 5, effector cells were harvested and tested for recognition of RMA-S or RMA-S.L^d cells incubated at 25°C overnight or RMA-S.L^d cells induced overnight at 25°C in the presence of 10^{-4} M MCMV or 10^{-4} M LCMV peptide.

the peptide requirement. This system is also the first in which the separate contribution of allogeneic MHC versus bound peptide can be addressed.

Other than the current study, the peptide ligand has been identified for only two alloreactive clones. An endogenous peptide ligand was recently identified for the well characterized 2C alloreactive L^d clone (13). Additionally a viral peptide, presumably cross-reactive with the endogenous peptide to which the clone was originally generated, was identified that would allow recognition of RMA-S cells by a K^b-specific alloreactive clone (33). This result differs from ours, however, in that we did not identify any clones generated to the viral peptide MCMV that were capable of cross-reacting with an endogenous peptide presented by L^d (discussed below).

Although we have demonstrated peptide-specific recognition in our system, we cannot exclude the existence of alloreactive CTL with very different recognition requirements. A study by Rötzschke et al (5) identified CTL with a variety of peptide requirements including CTL that recognize RMA-S cells induced at 25°C. These results suggest that alloreactive CTL may exist that are capable of recognizing empty MHC molecules. However, the ability of RMA-S cells to present endogenous molecules has been demonstrated by this group as well as others (34,35). Therefore, as stated by the authors, positive lysis is not assurance of recognition of empty molecules. Additionally, this group identified a line that recognized a range of peptides presented by K^b. However, as noted by the authors, this line may be polyclonal, as was our MCMV/L^d line, and therefore the existence of such a population remains unclear. If indeed peptide cross-reactive CTL exist, such a promiscuous peptide requirement could result from recognition of similar MHC conformational changes induced by peptide binding. Several families of peptides may exist that yield alternative MHC conformations upon binding (36, 37). When analyzed by cytolysis assays, it would appear that a given clone was capable of recognizing multiple peptides bound by the same MHC molecule, when in actuality only conformational determinants on the MHC were being directly recognized. In our analysis we did not identify any clones that displayed promiscuity in their peptide requirement, based on the following criterion. Given the complex array of endogenous peptides that are likely presented by L^d (38), it seems probable that the pool of endogenous peptides would encompass all of the possible conformation-inducing families, including a putative family to which MCMV would belong. Therefore lysis of nonpulsed targets by the MCMV/L^d-specific clones would have been observed. In no case did we identify clones capable of lysing both MCMV-pulsed T2.L^d targets and nonpulsed wild type L^d-expressing target cells. Although we cannot exclude the possibility of the existence of such alloreactive CTL, we feel it is unlikely that conformationally restricted CTL are a dominant portion of the alloresponse described in this study.

One of the central questions that has intrigued those studying allorecognition has been the vigorous in vitro primary response to alloantigens. Early studies demonstrated that the potency of the response was in part a result of a high frequency of alloantigen-specific precursors compared with peptide-specific, self-restricted precursors (39, 40). To explain the high frequency of alloantigen-specific precursors, an initial model proposed a requirement for recognition of residues exclusively within the MHC molecule (41). Thus every allogeneic MHC molecule could serve as an efficient recognition element for a responding alloreactive CTL. The resulting high determinant density would allow CTL displaying a wide range of affinities, i.e. those requiring few allogeneic MHC interactions, as well as those requiring many, to be effectively recruited. If indeed determinant density is not a limiting factor, then most, if not all, alloreactive CTL capable of responding would be activated, resulting in the high number of alloreactive CTL observed.

Our results suggest a high precursor frequency via an alternative model. Although this model has previously been postulated (42), only recently has methodology been developed that makes it readily testable. Recent data suggests that cells normally present a large number of distinct endogenous peptides complexed with self-MHC (38). If one proposes that a majority of these peptides are presented at levels capable of activating naive CTL, then CTL precursors of many different peptide specificities would be elicited during the course of a normal alloresponse. Therefore a high number of precursor CTL would be observed that were capable of responding to a given allogeneic MHC;

however, their individual peptide/MHC specificities would likely vary extensively. The ability to readily generate peptide-specific alloreactive MCMV/L^d and LCMV/L^d CTL strongly supports this model.

In summary we describe the generation of alloreactive CTL that are exquisitely peptide- and MHC-specific. It is noteworthy that the MCMV/L^d-specific alloreactive CTL could be generated from T cells selected from either the dm2 (H-2^{dm2}) or C57BL/6 (H-2^b) background. Neither endogenous peptides presented by L^d nor a panel of exogenous L^d peptide ligands could substitute for the MCMV peptide and allow for recognition by our MCMV/L^d alloreactive CTL clones. Additionally, the clones demonstrated a precise requirement for MCMV presentation by L^d as presentation by L^q was insufficient for recognition. Furthermore, the ability of our system to detect the differential contribution of peptide versus MHC in allorecognition demonstrates that residues within in the α helices as well as the β-pleated sheet are important for maximizing the efficiency of alloantigen recognition. This system is unique in that it can be utilized for the analysis of responses to the same peptide presented in the context of either self or foreign MHC molecules. Thus direct comparisons can be made to define the precise manner by which syngeneic versus allogeneic CTL interact with MHC/peptide complexes. This information may help in defining the details of alloreactive T lymphocyte generation as a consequence of positive and negative selection on self-MHC molecules.

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