The Journal of Immunology

RESEARCH ARTICLE | MAY 15 1992

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D B Crumpacker; ... et. al

J Immunol (1992) 148 (10): 3004–3011.
https://doi.org/10.4049/jimmunol.148.10.3004

0022-1767/92/14810-3004$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 148, 3004–3011, No. 10, May 15, 1992
Printed in U.S.A.

ROLE OF ENDOGENOUS PEPTIDES IN MURINE ALLOGENEIC CYTOTOXIC T CELL RESPONSES ASSESSED USING TRANSFECTANTS OF THE ANTIGEN-PROCESSING MUTANT 174xCEM.T2[1]

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One model to explain the high frequency of alloreactive T cells proposes that allogeneic MHC molecules are recognized together with host cell-derived peptides. A model system was developed to investigate the relevance of this mechanism by expression of H-2D^d or H-2L^d in 174xCEM.T2 (T2) cells. This human cell line contains a mutation in its Ag-processing pathway that should restrict the association of endogenous peptides with cell surface class I molecules. CTL generated by stimulating C57BL/6 (H-2^b) responder cells with H-2D^d or H-2L^d transfectants of the human B cell line C1R or the murine T cell lymphoma EL4 were assayed for their ability to recognize alloantigenic determinants on these transfectants. The major fraction of the H-2D^d-specific allogeneic CTL response, generated in a MLC or under clonal limiting dilution conditions, was composed of T cells that recognized H-2D^d expressed on C1R or EL4 cells, but failed to recognize this molecule on T2 cells. Clonal analysis indicated that approximately one-third of these CTL recognized determinants that were unique to H-2D^d expressed on C1R stimulator cells whereas the remainder recognized determinants that were also found on EL4 transfectants. Less than 10% of H-2D^d-reactive CTL recognized the T2 transfectant, and these clones also killed C1R-D^d and EL4-D^d. This result suggests that the great majority of H-2D^d-specific alloreactive CTL recognize determinants that are formed by a complex of H-2D^d with endogenous peptides that are absent or significantly reduced in T2 cells. Based on recognition of human or murine transfectants, these CTL exhibit some level of specificity for the structure or composition of the bound peptides. Examination of allogeneic CTL specific for H-2L^d revealed populations similar to those described for H-2D^d. In addition, a major new population was present that recognized determinants shared between C1R-L^d and T2-L^d but not present on EL4-L^d. These results are consistent with the idea that the alloreactive response to H-2L^d is also largely dependent on the presence of bound peptide. However, they also may indicate that the H-2L^d molecule expressed on T2 cells is occupied by one or more peptides that are shared with other human, but not murine, cells. The significance of these results to current models of alloreactivity is discussed.

Two models have been proposed to explain the high frequency of T cells that can respond to foreign MHC Ag. One model proposes that allogeneic TCR recognize the foreign MHC molecule in a manner that is dependent on presentation of peptides (1). In the case of T cells specific for class I molecules, this would be similar to the antigenic determinants recognized by virus-specific CTL and would involve the same pathways of Ag processing and presentation of endogenously synthesized cellular proteins. Support for this model has come from studies in which recognition by alloreactive T cells was shown to be dependent on the species of the cell expressing the MHC molecule (2, 3). These data suggested that the epitopes recognized consisted of complexes of the MHC molecule with one or more species-specific peptides. Further evidence that a fraction of alloreactive T cells may be dependent on the presentation of endogenous peptides comes from studies in which the epitope could be reconstituted using proteolyzed cellular extracts (4).

The alternative model proposes that MHC alloantigens are recognized based on their high density on the cell surface (5). This high determinant density model is distinct from the previous model in that recognition would not be specific for a peptide bound to the MHC molecule. Support for this model has come from the demonstration that an allogeneic T cell clone could be stimulated by a purified, apparently peptide-free, class I molecule (6). In addition, peptide nonspecific allorecognition by CTL has been reported (7). The models discussed need not be mutually exclusive and could each form a component of the alloreactive response. However, because all studies to date have used selected lines or clones, it is not clear what proportion of the alloreactive T cell repertoire might be represented by these alternative modes of recognition.

Attempts to determine the relevance of these two models for alloreactivity have profited by the generation of cell lines that are defective in peptide Ag processing

Received for publication November 12, 1991.
Accepted for publication February 26, 1992.
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[1] This work was supported by Public Health Service (PHS) Grants AI20963 (V.H.E.) and AI15775 (P.C.). D.B.C. is supported by PHS Training Grant CA09109.
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and presentation. The human mutant cell line 721.174 and its hybrid derivative 174xCEM.T2 (T2) have been shown to be defective in the presentation of class I restricted Ag introduced into the cytoplasm either by viral infection (8, 9) or by osmotic lysis of pinosomes (8). However, 721.174 is able to present an exogenously added viral peptide with greater efficiency than its normal parent (9). Similar results have been obtained using T2 (R. Henderson, unpublished observations). The defect in T2, 721.174, and similar mutants appears to result from the deletion of genes encoding a putative peptide transporter protein (9–11) in addition to a subunit of a multicatalytic proteasome complex thought to be important for peptide generation (12). 721.174 and T2 lack cell surface expression of HLA-B5 whereas HLA-A2 is expressed at reduced levels (9, 13). Transfection of T2 with either HLA or H-2 genes reveals an apparent differential transport of human and murine class I Ag in this processing mutant. The HLA-A2 and Aw68 molecules are somewhat "leaky" in their transport, but A3, B5, B7, and Bw58 are not expressed at the cell surface of T2 (14). In contrast, the H-2 Ag K^b, D^p, D^d, and L^d are transported in an apparently normal fashion (14, 15). Immunoprecipitation studies indicated that normal levels of H chain and β2m protein were synthesized in T2, yet the majority of H chains were not associated intracellularly with β2m (13). Together, these data suggest that these cell lines are defective in assembly and transport of peptide-H chain complexes. Consequently, the class I molecules that reach the cell surface should be largely free of bound peptides or at least associated with a severely restricted subset of such peptides.

In this study, transfectants of T2 expressing H-2D^d or L^d were used to examine the dependence of the murine alloantigenic response on the normal association of endogenous peptides with MHC molecules. It was determined to what extent murine alloreactive CTL generated by stimulation with transfectants of either human or murine cell origin could recognize the determinants present on T2-D^d and T2-L^d. This approach allowed an assessment of the fraction of peptide dependent CTL that are specific for peptides that differ between murine and human cells. Furthermore, the use of T2-D^d and T2-L^d as stimulators provided information as to the ability of the class I molecules expressed on these cell lines to generate an allo-response.

MATERIALS AND METHODS

Cell lines. The murine T cell lymphoma cell line EL4 (H-2^b) and the human B lymphoblastoid cell line Hmy2.C1R (C1R), selected for loss of the HLA-A,B gene products (16), were independently transfected with genomic clones of H-2D^d and H-2L^d by electroporation as previously described (17, 18). EL4, C1R, and 2PK-3 (H-2^d), a murine B cell lymphoma, were maintained in RPMI 1640 supplemented with 5% FCS (HyClone, Logan, UT), Serxtend (Hana Biologics, Irvine, CA), and 2 mM L-glutamine. The medium for transfectants of these cell lines also contained 300 μg/ml G418 (GIBCO, Grand Island, NY). The human BxT hybrid cell line T2, and the H-2D^d and H-2L^d transfectants of this cell line were maintained in ISCOVE'S Modified Dulbecco's Medium supplemented with 5% FCS, Serxtend, and 2 mM L-glutamine. The medium for T2 transfectants contained 200 μg/ml G418.

Generation of bulk alloantigenic CTL. C57BL/6 responder spleen cells (7×10^6) were stimulated with 1×10^5 irradiated (6000 rad) cells expressing H-2D^d or H-2L^d in 2 ml of RPMI 1640 containing 10% FCS, 15 mM HEPES, 50 μg/ml gentamycin, essential and nonessential amino acids, 5 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, and 2 mM L-glutamine (CM[4]). After 7 days, 1×10^6 cells were restimulated with 3×10^6 irradiated (3000 rad) syngeneic spleen cells and 1×10^5 irradiated stimulator cells in CM supplemented with 5 U/ml human rIL-2. The cultures were replated at weekly intervals under the same conditions and the responders were reduced to 5×10^5/well.

Assay of cytotoxic activity. A 4-h ^{51}Cr-release assay was used as described (19). Briefly, 2×10^3 ^{51}Cr-labeled target cells were incubated with the indicated number of CTL in a final volume of 150 μl in a V-bottom microtiter plate. Maximal release was determined in the presence of 2 N HCl. Percent specific ^{51}Cr release was determined by the formula:

$$100\% \times \frac{\text{experimental cpm released} - \text{spontaneous cpm released}}{\text{maximum cpm released} - \text{spontaneous cpm released}}$$

Where indicated, the T2 transfectants were incubated at 24°C for 22 h, labeled with ^{51}Cr for 2 h at 24°C and added to the microtiter plate. Cytotoxic activity was determined after 4 h at 37°C.

Limiting dilution analysis of cytotoxic activity. C57BL/6 spleen cells (3×10^4/well) were cultured in round bottom 96-well microtiter plates in 200 μl of CM to which 5 U/ml human rIL-2, 2.7×10^5 irradiated (3000 rad) syngeneic spleen cells and 1×10^4 irradiated (6000 rad) C1R cells expressing either H-2D^d or H-2L^d were added. After 7 days, the same amounts of fresh irradiated spleen cells and irradiated stimulator cells were added, and 4 days later aliquots (40 μl) of each well were assayed for cytotoxic activity. To accurately compare killing of different targets by the same CTL, significant lysis was defined as >20% specific release. This value is higher than the more conventional cutoff of 3 SD above the mean spontaneous release value from eight replicate wells containing only target cells.

FACS analysis of transfectants. Cell surface expression of the transfected H-2D^d and H-2L^d gene products was assessed by flow cytometry using saturating amounts of appropriate mAb. The mAb used to detect cell surface expression of H-2D^d were 34-1-2 (α1), 34-5-8 (α2), and 34-2-12 (α3). Those used to detect H-2L^d were 30-5-7 ($\alpha 1 \alpha 2$) and 28-14-8 (α3). Where indicated, the T2 transfectants were incubated at room temperature (24°C) for 24 h before FACS analysis.

RESULTS

Comparison of class I expression on transfectants. Although murine class I molecules are transported to the cell surface in the human processing mutant T2, their level of expression is generally lower than that on other H-2 transfectants (14). To compare T2 with these other cells as targets for CTL, conditions were established to increase the cell surface expression of H-2 molecules. The levels of expression of H-2D^d on transfectants of the human B lymphoblastoid cell line C1R (C1R-D^d) and the murine T cell lymphoma EL4 (EL4-D^d) were similar (Table I). However, expression on T2 transfectants was only 12 to 17% of the level on C1R-D^d. After incubation for 24 h

TABLE I

Surface expression of H-2D^d on transfected cell lines[a]

Cell Line	Temperature (°C)	Mean Fluorescence		
		34-1-2	34-5-8	34-2-12
C1R-D^d	37	493 (1.0)[b]	301 (1.0)	306 (1.0)
EL4-D^d	37	[c]	192 (0.64)	298 (0.97)
T2-D^d	37	58 (0.12)	42 (0.14)	51 (0.17)
T2-D^d	24[d]	146 (0.3)	90 (0.3)	120 (0.4)
Fold increase[e]		2.5	2.1	2.4

[a] Mean fluorescence values for treatment with fluorescein-conjugated antibody alone were subtracted from experimental values to give corrected mean fluorescence values. Data presented represent the averages of between 6 and 11 separate experiments.

[b] Mean fluorescence of H-2D^d on the C1R transfectant was assigned a value of 1.0 and values in parentheses presented for the EL4 and T2 transfectants reflect levels of expression relative to C1R-D^d.

[c] The mAb 34-1-2 recognizes H-2K^b present on EL4, and could not be used to quantitate D^d expression on this transfectant.

[d] T2-D^d was incubated at 24°C for 24 h before FACS analysis.

[e] The fold increase was calculated from the average values of fluorescence intensity presented.

[4] Abbreviation used in this paper: CM, complete medium.

at 24°C, the expression on T2 increased to approximately 30 to 40% of the level present on C1R-D^d and EL4-D^d. This increase was seen using antibodies specific for the α1 (34-1-2), α2 (34-5-8), and α3 (34-2-12) domains of H-2D^d and reflects, on average, a 2.3-fold increase in expression of these epitopes. The level of expression of H-2L^d on T2 transfectants at 37°C was comparable to that found on C1R-L^d and EL4-L^d (Table II) and could be increased, on average, by 2.7-fold after incubating T2-L^d at 24°C for 24 h. Similar increases were detected using mAb directed against the α1α2 (30-5-7) and α3 (28-14-8) domains. Under these conditions, the expression of H-2L^d on T2 cells was higher than that found on C1R, and about 56% of that found on EL4 transfectants.

Maintenance of low temperature-induced epitopes at 37°C. The increased surface expression of the 34-5-8 (α2) and 34-2-12 (α3) epitopes induced by incubation of T2-D^d at 24°C was relatively stable after shifting the temperature back to 37°C for up to 4 h (Fig. 1*A*). These results establish that T2 cells can be induced to express H-2D^d molecules at 30 to 40% of the levels found on other transfectants used in this study, and that this level of expression is maintained under the conditions used for ^{51}Cr release assays. In contrast, the induced 30-5-7 (α1α2) and 28-14-8 (α3) epitopes stabilized on H-2L^d decline more rapidly on the surface of T2 during the first 2 h of incubation at 37°C (Fig. 1*B*). Nonetheless, expression on T2 could be elevated for up to 2 h after shifting to 37°C.

TABLE II

Surface expression of H-2L^d on transfected cell lines[a]

Cell Line	Temperature (°C)	Mean Fluorescence	
		30-5-7	28-14-8
C1R-L^d	37	18 (1.0)[b]	34 (1.0)
EL4-L^d	37	50 (2.8)	[c]
T2-L^d	37	11 (0.6)	30 (0.9)
T2-L^d	24[d]	28 (1.6)	86 (2.5)
Fold increase[e]		2.5	2.9

[a] Mean fluorescence values for treatment with fluorescein-conjugated antibody alone were subtracted from experimental values to give corrected mean fluorescence values. Data presented represent the averages of between 4 and 10 separate experiments.

[b] Mean fluorescence of H-2L^d on the C1R transfectant was assigned a value of 1.0 and values in parentheses presented for the EL4 and T2 transfectants reflect levels of expression relative to C1R-L^d.

[c] The mAb 28-14-8 recognized H-2D^b present on EL4 and could not be used to quantitate H-2L^d expression on this transfectant.

[d] T2-L^d was incubated at 24°C for 24 h before FACS analysis.

[e] The fold increase was calculated from the average values of fluorescence intensity presented.

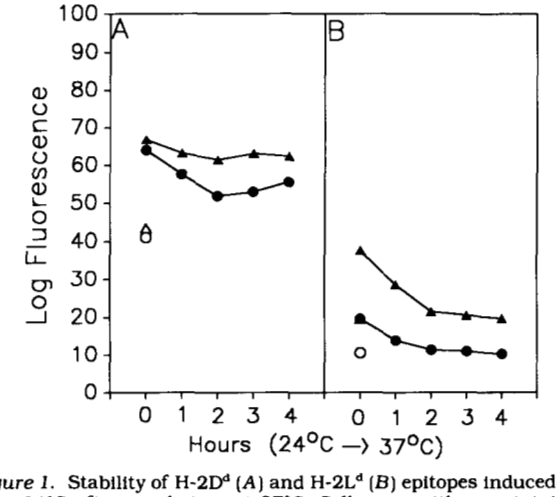

Figure 1. Stability of H-2D^d (*A*) and H-2L^d (*B*) epitopes induced on T2 cells at 24°C after incubation at 37°C. Cells were either maintained at 37°C (*open symbols*) or incubated at 24°C for 24 h (*closed symbols*) before FACS analysis. mAb used to detect determinants on H2-D^d were 34-5-8 (●) and 34-2-12 (▲) and those used to detect H2-L^d were 30-5-7 (●) and 28-14-8 (▲). Mean fluorescence values for treatment with fluorescein-conjugated antibody alone were subtracted from experimental values to give corrected mean fluorescence values.

Recognition of T2 transfectants by bulk allogeneic CTL. Transfectants of the human peptide processing mutant T2 expressing murine class I Ag were used to examine the dependence of the murine allogeneic response on endogenous peptides. Alloreactive CTL were initially generated from C57BL/6 (H-2^b) splenic responder cells by stimulation with H-2D^d or H-2L^d transfectants of EL4, C1R, or T2. Both murine EL4 and human C1R transfectants expressing H-2D^d-stimulated allogeneic CTL that specifically recognized EL4-D^d and another H-2D^d-expressing cell, 2PK-3 (Fig. 2, *A* and *B*), but failed to recognize untransfected EL4 cells (data not shown). However, stimulation with T2-D^d failed to generate CTL that recognized EL4-D^d or 2PK-3. In addition, none of the transfectants, including T2, stimulated CTL that were able to recognize T2-D^d target cells (Fig. 2*C*). Similar results were obtained with H-2L^d transfected cells (Fig. 2, *D*, *E*, and *F*). Thus, T2 transfectants were unable to stimulate primary in vitro allogeneic CTL that could recognize either the stimulator cells or other cells expressing these class I molecules.

Bulk CTL established by stimulation with these transfectants were restimulated weekly for an additional 2 wk in an attempt to grow out T2-reactive cells. However, cultures restimulated with either transfectant of T2 failed to exhibit any significant CTL activity after this time (data not shown). However, bulk cultures restimulated with either C1R-D^d or EL4-D^d did exhibit low but significant lysis of T2-D^d after this time (Fig. 3C). The failure of T2-D^d to be lysed as efficiently as C1R-D^d or

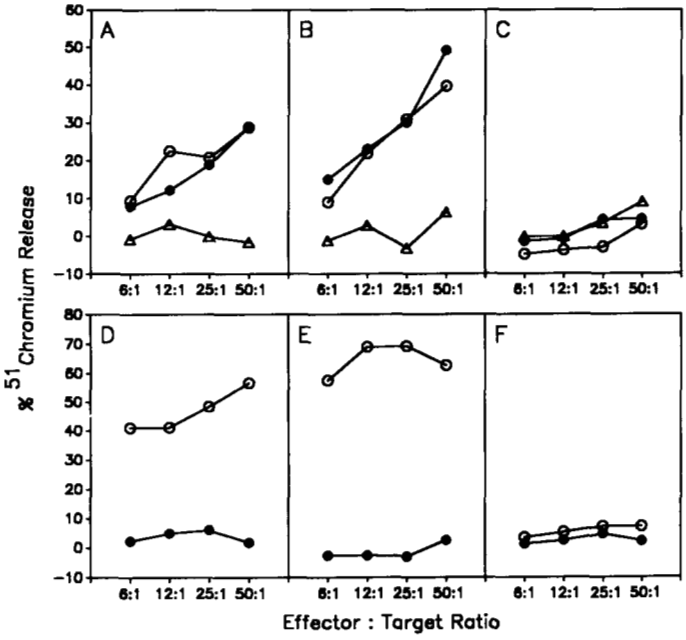

Figure 2. Allogeneic CTL generated from a bulk mixed lymphocyte culture by stimulation with either EL4-D^d (○), C1R-D^d (●), or T2-D^d (△) were assayed on day 5 of culture against EL4-D^d (*A*), 2PK-3 (*B*), and T2-D^d (*C*). CTL generated by stimulation with either EL4-L^d (○) or T2-L^d (●) were assayed on day 5 of culture against EL4-L^d (*D*), 2PK-3 (*E*), and T2-L^d (*F*).

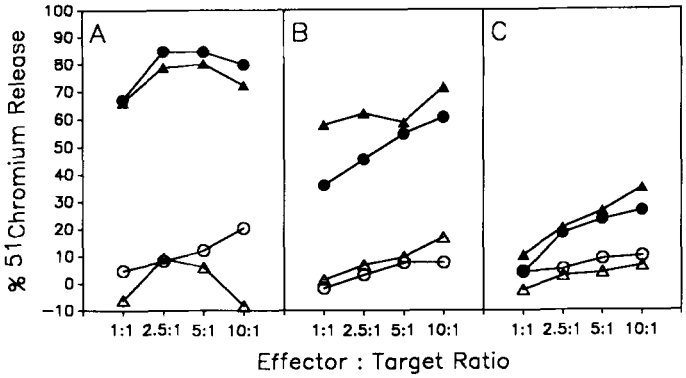

Figure 3. Allogeneic CTL generated from bulk mixed lymphocyte cultures by stimulation with either EL4-D^d (*circles*) or C1R-D^d (*triangles*) were assayed during the 3rd wk of culture against either untransfected (*open symbols*) or H-$2D^d$ transfected (*closed symbols*) target cells. The transfected and untransfected pairs were: *A*, EL4-D^d and EL4; *B*, C1R-D^d and C1R; *C*, T2-D^d and T2. T2-D^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 24°C and addition to the assay.

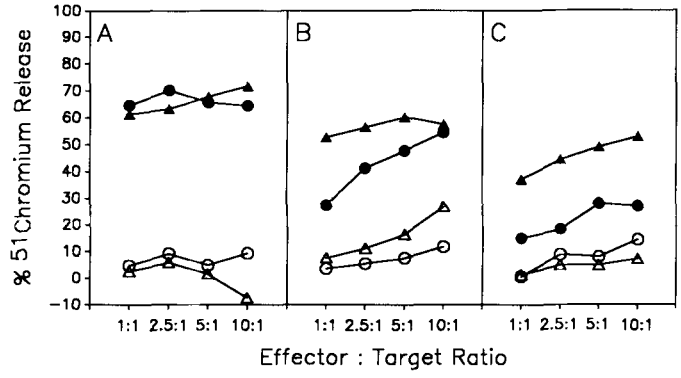

Figure 4. Allogeneic CTL generated from bulk mixed lymphocyte cultures by stimulation with either EL4-L^d (*circles*) or C1R-L^d (*triangles*) were assayed during the 3rd wk of culture against either untransfected (*open symbols*) or transfected with H-$2L^d$ (*closed symbols*). The transfected and untransfected pairs were: *A*, EL4-L^d and EL4; *B*, C1R-L^d and C1R; *C*, T2-L^d and T2. T2-L^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 24°C and addition to the assay.

EL4-D^d is unlikely to be due to low surface expression, because the surface expression induced at 24°C is maintained during the course of the ^{51}Cr release assay (Fig. 2*A*). Instead, the low level of lysis is probably due to the out-growth of a population of precursors that recognize determinants expressed on this cell line. Both EL4-D^d and C1R-D^d target cells were killed at comparable levels by cultures restimulated with either of these two transfectants (Fig. 3, *A* and *B*). Within the limits of this analysis, this indicates that the species of origin of the target cell does not influence the specificity of this response. Given the low level of lysis of T2-D^d by the same CTL, these results suggest that the epitopes recognized are dependent upon peptides that are not found on T2 cells but may be shared between the nondefective human and murine stimulator cell lines.

As with the H-$2D^d$-specific allogeneic CTL, cultures restimulated for 3 wk with either human or murine H-$2L^d$ transfectants gave comparable lysis of EL4-L^d and C1R-L^d (Fig. 4, *A* and *B*). Interestingly, whereas T2-L^d was still recognized poorly by the EL4-L^d-restimulated line, it was recognized quite well when the stimulator cell was of human origin (Fig. 4*C*). These results were consistent with the idea that the response to H-$2L^d$ was peptide dependent, at least when the stimulating cell was of murine origin. However, the unexpectedly high level of recognition of T2-L^d observed using C1R-L^d-restimulated CTL suggested that the peptide dependence of this response could be influenced by the stimulator cell used.

Recognition of T2-D^d by allogeneic CTL generated under clonal limiting dilution conditions. The data from the bulk cultures suggested that the predominant population of H-$2D^d$-alloreactive CTL recognized epitopes that were dependent on the presence of an endogenous peptide. However, this type of data is difficult to quantitate, and give no information about the specificity of individual CTL. Consequently, H-$2D^d$-alloreactive CTL were generated under limiting dilution conditions where the fraction of positive wells was low enough to give confidence that the responses were clonal. Split well analysis using C1R-D^d, EL4-D^d, and T2-D^d target cells allowed the dependence of these CTL on endogenous peptides, and their ability to distinguish between peptides of either human or murine origin, to be assessed. The human cell C1R-D^d, rather than EL4-D^d, was used as a stimulator for these experiments to eliminate the possibility that a fraction of CTL would fail to recognize T2 because of suboptimal interactions between murine T cell accessory molecules and their ligands on human cells.

A representative experiment (Fig. 5*A*) shows that the major fraction of these C1R-D^d specific CTL (defined as giving >20% lysis of C1R-D^d and <19.5% lysis of C1R) recognize not only the stimulator cell, but EL4-D^d as well. In addition, wells were identified that lysed C1R-D^d alone, or C1R-D^d, EL4-D^d, and T2-D^d (Fig. 5*B*). Cytotoxicity data from six separate experiments are summarized in Table III. Among individual experiments, there is some variation in the fraction of cells associated with each reactivity pattern. However, summation of responses involving 135 C1R-D^d-specific wells reveals that this allogeneic response consists primarily of CTL that recognize either C1R-D^d alone (31%) or both C1R-D^d and EL4-D^d (57%) but fail to recognize T2-D^d. Similar to the data obtained from bulk CTL cultures, these results indicate that the major fraction of this response is composed of T cells that recognize a complex of H-$2D^d$ and endogenous peptides. In addition, the recognized peptide can either be unique to the human stimulator cell or shared with murine cells. A small but significant component of the response to C1R-D^d (9%) consists of CTL that recognize C1R-D^d, EL4-D^d, and T2-D^d.

Recognition of T2-L^d by allogeneic CTL generated under clonal limiting dilution conditions. When the response stimulated by C1R-L^d was examined under clonal limiting dilution conditions, patterns of reactivity similar to those seen in the H-$2D^d$-specific response were observed (Fig. 6*A*). However, a fraction of wells showed reactivity on the human transfectants C1R-L^d and T2-L^d but not EL4-L^d (Fig. 6*B*). This pattern was not observed at significant levels in the H-$2D^d$ response, but reinforces the data from bulk CTL which indicated that stimulation with C1R-L^d generated a very high response against T2-L^d (Fig. 4*C*). Data from five separate limiting dilution experiments reveal that a large proportion (50%) of the wells demonstrated lysis of C1R-L^d alone, whereas only a minor fraction (10%) exhibited lysis of C1R-L^d, EL4-L^d, and T2-L^d (Table IV). However, the fraction of wells exhibiting lysis of both C1R-L^d and EL4-L^d but not T2-L^d (6%) was greatly diminished compared to the H-$2D^d$ re-

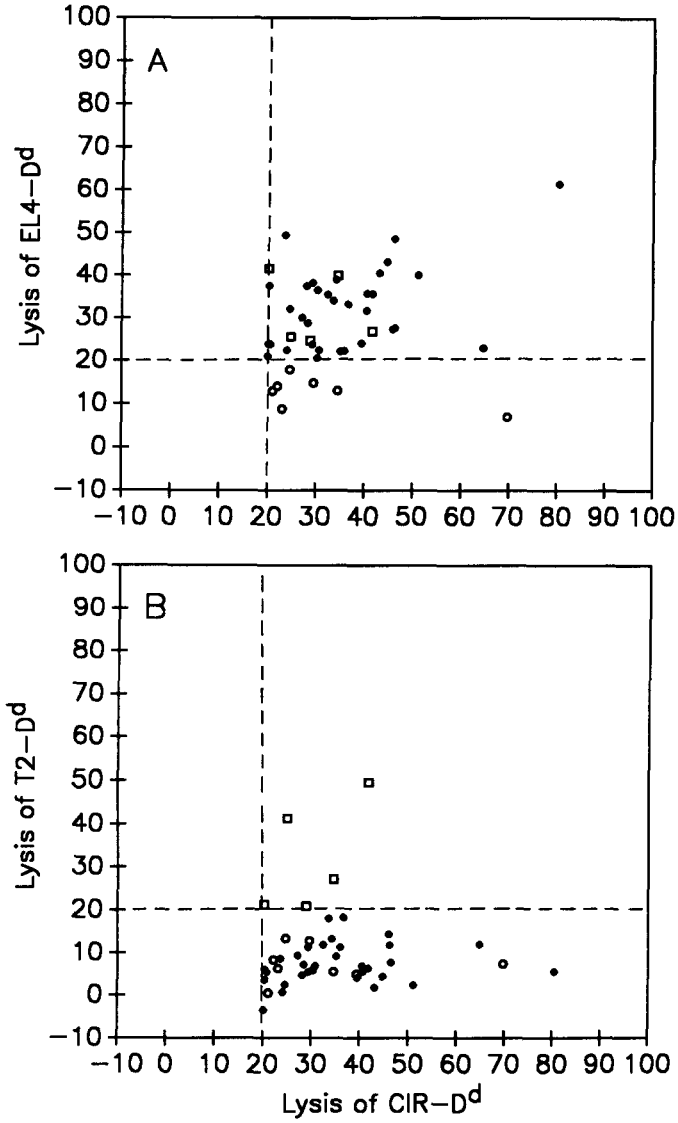

Figure 5. Allogeneic CTL generated by stimulation with C1R-D^d under clonal limiting dilution conditions were assayed by split well analysis on C1R, C1R-D^d, EL4-D^d, and T2-D^d target cells on day 11 of culture. Wells exhibiting positive lysis of the untransfected C1R parent cell line were excluded from this analysis. *Dotted lines* indicate significant lysis limits. T2-D^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 24°C and addition to the assay. *A* and *B* show comparative lysis of C1R-D^d and either EL4-D^d or T2-D^d, respectively, whereas complete patterns of cytotoxic activity are specified by the use of symbols: C1R-D^d alone (○); C1R-D^d and EL4-D^d (●); C1R-D^d, EL4-D^d, and T2-D^d (□).

TABLE III

Pattern of lysis for allogeneic CTL stimulated with C1R-D^d under clonal limiting dilution conditions[a]

Total wells analyzed Total CIR-D^d specific wells	Expt. 1	2	3	4	5	6	Total	Percent of Response
	48	48	48	384	384	384	1296	
	3	13	4	30	49	36	135	
CIR-D^d only	2	7	2	6	9	16	42	31
CIR-D^d and EL4-D^d	1	2	1	22	35	16	77	57
CIR-D^d, EL4-D^d and T2-D^d	0	1	0	2	5	4	12	9
CIR-D^d and T2-D^d	0	3	1	0	0	0	4	3

[a] Positive wells were selected based on values that were at least 3 SD above the mean spontaneous release values for each target. Wells that exhibited positive lysis of the untransfected C1R cell line were considered nonspecific and were not included in this analysis. T2-D^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 22°C and addition to the assay.

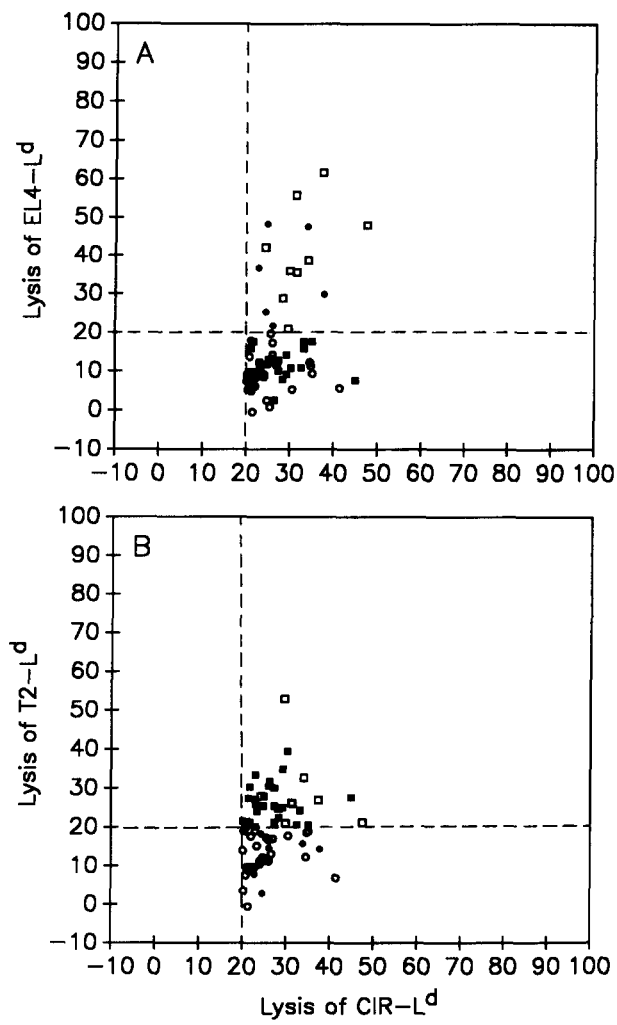

Figure 6. Allogeneic CTL generated by stimulation with C1R-L^d under clonal limiting dilution conditions were assayed by split well analysis on C1R, C1R-L^d, EL4-L^d, and T2-L^d target cells on day 11 of culture. Wells exhibiting positive lysis of the untransfected C1R parent cell line were excluded from this analysis. *Dotted lines* indicate significant lysis limits. T2-L^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 24°C and addition to the assay. *A* and *B* show comparative lysis of C1R-L^d and either EL4-L^d or T2-L^d, respectively, although complete patterns of cytotoxic activity are specified by the use of symbols: C1R-L^d alone (○); C1R-L^d and EL4-L^d (●); C1R-L^d, EL4-L^d and T2-L^d (□); C1R-L^d and T2-L^d (■).

TABLE IV

Pattern of lysis for allogeneic CTL stimulated with C1R-L^d under clonal limiting dilution conditions[a]

Total wells analyzed Total CIR-L^d specific wells	Expt. 1	2	3	4	5	Total	Percent of Response
	48	48	384	384	384	1248	
	2	14	69	6	17	108	
CIR-L^d only	0	13	27	4	10	54	50
CIR-L^d and EL4-L^d	0	0	6	0	0	6	6
CIR-L^d, EL4-L^d and T2-L^d	1	0	10	0	0	11	10
CIR-L^d and T2-L^d	1	1	26	2	7	37	34

[a] Positive wells were selected based on values that were at least 3 SD above the mean spontaneous release values for each target. Wells that exhibited positive lysis of the untransfected C1R cell line were considered nonspecific and were not included in this analysis. T2-L^d was incubated at 24°C for 22 h before labeling with ^{51}Cr for 2 h at 22°C and addition to the assay.

sponse (57% in Table III). These results indicate that the allogeneic response against H-2L^d is still largely peptide dependent, and that the majority of these CTL is specific

for peptides that differ between murine and human cells. Also in contrast to the results observed with H-2D^d, there is a high percentage (34%) of CTL that recognize C1R-L^d and T2-L^d but not EL4-L^d. The failure to recognize EL4-L^d suggests that these CTL are peptide dependent. Their recognition of T2-L^d could represent binding to H-2L^d of one or more peptides derived from human cells via an alternative processing pathway. Such peptides might also have been released from C1R and T2 and then bound exogenously by H-2L^d.

DISCUSSION

We have used transfectants of the human cell line T2 to examine the importance of endogenous, class I MHC-associated peptides in the allogeneic CTL response to H-2D^d and L^d. The available evidence has led to the suggestion that class I molecules expressed on the surface of T2 cells should be largely free of such peptides due to a defect in the endogenous Ag-processing pathway (9–12). Although H-2L^d and H-2D^d molecules expressed on T2 have not been examined, K^b and D^b molecules expressed on T2 do in fact contain severely reduced quantities of peptides[5]. In addition, HLA-A2 expressed on T2 is associated with a normal amount of peptide but this is comprised of only a small number of peptide species[6]. Thus, failure to kill these T2 transfectants reflects the extent to which epitopes recognized by allogeneic CTL are dependent on the presence of a peptide in the class I binding site. Furthermore, recognition by these CTL of nondefective human and murine cells transfected with the same class I molecules was compared. Inasmuch as murine and human cells are likely to differ in the structure and composition of endogenous peptides associated with H-2D^d and L^d, this approach allowed a minimum estimate of the fraction of CTL that were peptide specific.

The data indicated that T2-D^d was recognized very poorly by allogeneic bulk CTL specific for the H-2D^d molecule. In addition, T2-D^d was unable to stimulate a significant response against either itself or other H-2D^d-expressing cell lines. Finally, a large majority (88%) of murine allogeneic CTL analyzed under clonal limiting dilution conditions failed to recognize T2-D^d. Incubation at 24°C was used to increase the level of expression on T2-D^d to a level comparable to that of C1R-D^d and EL4-D^d and the induced surface expression was relatively stable during the course of the 4-h ^{51}Cr release assay. Therefore, failure to kill T2-D^d cannot be attributed to low surface expression of the H-2 Ag. Rather, all of the data obtained suggest that the major fraction of this allogeneic response is composed of T cells that recognize a complex of H-2D^d and endogenous peptides that are absent or poorly expressed on the surface of T2. This conclusion is further supported by the observation that approximately one-third of the CTL identified under limiting dilution conditions that failed to recognize T2-D^d were also unable to recognize H-2D^d expressed on a cell of a different species, consistent with their recognition of one or more peptides associated with H-2D^d on the surface of human cells, but absent from murine cells.

A similar analysis of human allogeneic responses to HLA-A2 expressed on PBL has shown that 85% of the CTL were unable to recognize this class I molecule expressed on T2 cells (19a). In addition, Heath et al. (4) have recently shown that 17 of 19 H-2K^b-specific allogeneic CTL clones did not recognize T2 transfectants expressing H-2K^b. Although this is a smaller and possibly nonrandom sampling of this response, these workers did establish that the epitopes recognized by 12 of these clones could be reconstituted by addition of a protein digest, thus confirming their peptide dependency. Finally, the reconstitution of different epitopes by different fractions of this digest suggested that such CTL were peptide specific as well as peptide dependent. The results of these studies with three different class I molecules reinforce one another and lead to the conclusion that the epitopes recognized by the great majority of allogeneic CTL on most class I MHC molecules are dependent on the presence of a bound peptide. At least one-third of such CTL are also sensitive to the structure of the peptide. However, the range of peptide structures that are effective in epitope formation remains to be established.

As with H-2D^d-specific CTL, the majority of allogeneic CTL generated under clonal limiting dilution conditions using C1R-L^d as a stimulator cell recognized C1R-L^d alone or C1R-L^d and EL4-L^d, but failed to recognize T2-L^d. Although the fraction of the total response with these reactivity patterns (56%) was smaller than for H-2D^d, these results nonetheless suggest that a significant component of this allogeneic response is peptide dependent. Furthermore, more than 90% of the CTL in this group recognized C1R-L^d only, indicating specificity for peptides expressed on the surface of this human cell line. Surprisingly however, a major fraction (44%) of CTL analyzed under clonal limiting dilution conditions recognized C1R-L^d and T2-L^d. Bulk CTL stimulated with H-2L^d-expressing human cells, but not murine cells, also showed a high level of reactivity on T2-L^d. In clonal limiting dilution responses, three-quarters of the CTL that recognized C1R-L^d and T2-L^d failed to recognize EL4-L^d. These results cannot be due to the level of expression of H-2L^d, because this is highest on EL4. It is possible that these CTL are specific for epitopes on H-2L^d that are sensitive to differences between murine and human β2-m. However, studies in other systems have not detected such an effect (2, 23–26). It seems most likely that the data reflect the recognition of H-2L^d-associated peptides that are found on both C1R and T2 cells, but are absent from EL4.

Additional evidence suggesting that some class I molecules expressed on T2 may be associated with some peptides comes from work with murine T cell clones (designated AH), that recognize HLA-A2 when expressed on the surface of human, but not murine cells (27). These AH clones are not sensitive to changes in β2-m or the presence or structure of the class I-associated carbohydrate side chain, and it has been suggested that they recognize human peptides associated with HLA-A2. Nonetheless, these AH clones recognize T2 cells expressing HLA-A2 as well as normal HLA-A2+ cells (R. Henderson, manuscript in preparation). This is consistent with the observation that HLA-A2 molecules expressed on T2

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do in fact contain high levels of a limited spectrum of peptides (20, 21).

The high percentage of H-2L^d-specific CTL that recognize this molecule on T2 cells, and the high percentage that are specific for C1R-L^d only, may also reflect the unique properties of this class I molecule. These include weak association with β2m, slow intracellular transport, and low cell-surface expression (25, 26). These properties have been suggested to reflect a low level of association with endogenous peptide, and binding studies suggest that there is a significant reservoir of peptide-free H-2L^d molecules on the surface of normal cells (27, 28). (Peptide-free and bound forms are distinguishable by their reactivity with the 30–5–7 and 28–14–8 mAb. However, because these epitopes exhibit a parallel increase in expression on T2 cells incubated at 24°C, we do not believe that this treatment has changed their relative distribution.) Although most class I molecules are thought to be largely occupied by endogenous peptides in the endoplasmic reticulum or early Golgi compartments (29, 30), the presence of significant numbers of peptide-free H-2L^d molecules in more distal compartments may allow them to access peptides generated via alternative processing mechanisms, or that are present in the extracellular space. One additional consequence of this would be a more restricted distribution of H-2L^d-associated peptides on the surface of normal cells. This could in turn account for the higher degree of species-specific recognition observed in the H-2L^d response as compared to the H-2D^d response.

Although the data from this study suggest that the great majority of allogeneic CTL recognized H-2D^d and H-2L^d as a complex with an endogenous peptide, a minor but significant fraction (9–10%) recognize these class I molecules whether they are expressed on a human or murine cell line or a cell line defective in peptide Ag processing. Inasmuch as it is not clear how absolute the Ag-processing defect is in T2 cells, the nature of the epitopes recognized by this component of the response is unclear. It may represent CTL that recognize class I Ag in a manner that is insensitive to the presence or absence of endogenous peptides. However, it may represent CTL that recognize peptides generated by the alternative mechanisms outlined above, and shared by both human and murine cells. Regardless of which of these two explanations holds, it is clear that most, and perhaps all, of these allogeneic CTL are dependent on the presence of peptides bound to class I MHC molecules. Although a major fraction is influenced by the structure of the bound peptide, consistent with the model for alloreactivity proposed by Matzinger and Bevan (1), it is still possible that a fraction, although peptide dependent, is not influenced by peptide structure. Inasmuch as this distinction was not made in the original high determinant density model, our results cannot be said to absolutely support either of the previously proposed models for alloreactivity to the exclusion of the other. Nonetheless, the necessity for a large population of low affinity CTL to account for the magnitude of the alloreactive response would seem to be obviated by our results. Instead, further refinements in this system will be directed toward determination of the contribution of peptide structure to epitope formation.

Acknowledgments. We thank Dr. Shu Man Fu for the use of the FACSCAN and Marie Burton for help in the preparation of this manuscript.

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