DIRECT DEMONSTRATION THAT CYTOTOXIC T LYMPHOCYTES RECOGNIZE CONFORMATIONAL DETERMINANTS AND NOT PRIMARY AMINO ACID SEQUENCES*

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The notion that alloimmune as well as self-restricted cytotoxic T lymphocytes (CTL) recognize conformational determinants on class I major histocompatibility complex (MHC) molecules rather than primary amino acid (AA) sequences is based on the following arguments. First, an increasing body of evidence shows that the repertoires of alloimmune and self-restricted CTL overlap and thus recognize similar target structures on class I MHC molecules (1-3). This finding is explained best by assuming that self-restricted CTL recognize antigen-induced conformational changes in self-class I molecules rather than foreign antigen itself (4). Indeed, antigen-specific CTL can be blocked easier with anti-H-2 antibody than with antibody to the foreign antigen (5, 6), and virus-specific CTL are more cross-reactive than virus-specific antibody (6). Second, analysis of alloimmune CTL directed against mutant H-2K\(^b\) molecules has provided a more direct argument for recognition of conformational H-2 determinants by CTL. Bulk CTL or CTL clones generated against a particular H-2K\(^b\) mutant cross-reacted with other K\(^b\) mutants bearing unrelated AA substitutions in completely different sites of the H-2K\(^b\) molecule (4, 7).

We have now asked whether B6 anti-bm1 CTL, exclusively directed against antigens created by AA substitutions at positions 152, 155, and 156 of the H-2K\(^b\) molecule (8, 9), detect the same antigens in the H-2L\(^d\) molecule, which is structurally identical with the H-2K\(^bm\) molecule from positions 146-162 (8-11), thus including all three AA substitutions, but differs considerably elsewhere. The answer is no, thus providing strong direct evidence for the notion that CTL do not recognize primary AA sequences, but conformational determinations on class I MHC molecules.

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Materials and Methods

Animals. All mice were bred at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Generation of Alloimmune CTL. Responder spleen cells (10^8) were cocultured with irradiated (2,000 rad) spleen cells (10^8) as stimulator cells in 80 ml of culture medium for 5 d at 37°C in humidified air with 5% CO2. The culture medium consisted of Iscove’s modified Dulbecco’s medium (IMDM) with 10% pooled human serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 2-mercaptoethanol (2 x 10^-5 M).

Cell-mediated Lymphocytotoxicity (CML). Varying numbers of effector cells were added to 3 x 10^4 Na_251CrO_4 (~Cr)-labeled target cells in 0.2 ml IMDM supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY) in wells of round-bottom microtiter plates and incubated for 4 h at 37°C in humidified air with 5% CO2. After incubation, the supernatant was collected with the Titertek Supernatant Collection System (Flow Laboratories, Inc., McLean, VA). As target cells we used Con A-induced (2.5 µg/ml) lymphoblasts. The percentage of specific ~Cr release was calculated by the formula:

\[ \text{% specific lysis} = \frac{\text{cpm experimental well} - \text{background ~Cr release}}{\text{cpm 5% saponin release} - \text{background ~Cr release}} \times 100. \]

Background ~Cr release was taken to be the release in the presence of responder spleen cells cocultured with irradiated syngeneic spleen cells. The standard error of triplicate cultures was always <3% specific ~Cr release.

Adsorption of CTL on spleen-cell monolayers was performed as described before (12).

Results

Cross-reactivity of B6 Anti-bm1 CTL Against Target Cells of Various H-2 Haplotypes Including H-2d. B6 (H-2^b) mice are tolerant of all bm1 structures except for new antigens created by the AA substitutions at positions 152, 155, and 156 of the H-2K^bm1 molecule (8, 9). Because the L^d molecule is structurally identical with the K^bm1 molecule in the AA positions 146-162 (10, 11), including AA substitutions responsible for all antigenic changes in bm1 against which B6 is allowed to react, it was of interest to test whether L^d-bearing target cells were lysed to the same extent as bm1 target cells. The results shown in Fig. 1 indicate that this is not the case. Although CTL cross-reactivity against B10.D2 (H-2^d) as well as B10.0R (H-2^k), B10.G (H-2^q), B10.R III (H-2^r), and B10.M (H-2^f) target cells was apparent, the level of lysis was much less than against the sensitizing type bm1 target cells. Low and inconsistent levels of cross-kill were observed against B10.S (H-2^s) and C3H.NB (H-2^p) targets (Fig. 1).

L^d-bearing Monolayers Fail to Adsorb the Activity of B6 Anti-bm1 CTL Against bm1 Target Cells. Because of the structural relationship between K^bm1 and L^d referred to above, it was of interest to test whether L^d-bearing B10.D2 monolayers could adsorb all CTL activity against bm1 target cells. The results represented in Fig. 2A show that they failed to do so. In control experiments, adsorption of anti-bm1 CTL to bm1 monolayers led to strong reduction of CTL activity against bm1 and to complete elimination of all cross-reactivity against H-2^qd, H-2^k, and H-2^f target cells (data not shown). Adsorption to B6 monolayers did not influence the CTL reaction pattern (data not shown). Thus, a major cell population among B6 anti-bm1 CTL is uniquely directed against K^bm1 and cannot be adsorbed to L^d-bearing monolayers, even though the reactivity against B10.D2 targets was almost completely removed (Fig. 2A).

Recognition of H-2L^d by a Subset of B6 Anti-bm1 CTL. Although the anti-H-2^d
activity of B6 anti-bm1 CTL is clearly distinct from a major CTL population uniquely directed against bm1, it was still of interest to establish whether the anti-H-2<sup>d</sup> activity is directed against H-2L<sup>d</sup>. In direct lysis experiments, dm2 (L<sup>d</sup> loss mutant) target cells were lysed to the same extent as B10.D2 target cells (data not shown). Adsorption experiments indicated that B10.D2 monolayers adsorbed all CTL activity against both B10.D2 and dm2 targets (Fig. 2A), whereas dm2 monolayers adsorbed the activity against dm2 but not against B10.D2 targets (Fig. 2B). Thus, the anti-H-2<sup>d</sup> activity is distinct from the unique anti-bm1 CTL population and can be ascribed to at least two other CTL populations, one directed against L<sup>d</sup> and another directed against H-2<sup>d</sup> minus L<sup>d</sup>.

Further Identification of CTL Subpopulations Included in B6 Anti-bm1 CTL. By means of monolayer adsorption, additional CTL subpopulations included within B6 anti-bm1 CTL were identified.

(A) Adsorption with H-2<sup>d</sup> did not remove CTL activity against B10.BR (H-2<sup>k</sup>) (Fig. 2A). This anti-H-2<sup>k</sup> population is distinct from the unique anti-bm1 subset because adsorption onto B10.BR monolayers strongly reduced CTL activity against B10.BR but not against bm1 target cells (Fig. 2C). As expected, the adsorption onto B10.BR did not reduce the activity against B10.D2 and dm2 (Fig. 2C).

(B) The anti-H-2<sup>k</sup> CTL population was shown to consist of at least two subsets. One is directed against K<sup>k</sup> because after absorption onto C3H.OH (K<sup>d</sup> D<sup>k</sup>) monolayers CTL activity against B10.A (4R) (K<sup>k</sup> D<sup>k</sup>) and B10.BR remained (Fig. 2D). The other is directed against D<sup>k</sup> because B10.A (4R) monolayers failed to remove CTL activity against B10.BR target cells (Fig. 2E). The latter adsorption also failed to remove activity against B10.AKM (K<sup>k</sup>D<sup>k</sup>) targets. This indicates that the anti-H-2<sup>k</sup> cross-reaction includes a D<sup>k</sup> component distinct from the H-2K<sup>k</sup> population (Fig. 2E). It was not investigated whether the anti-D<sup>k</sup> population is distinct from the anti-D<sup>k</sup> population.
FIGURE 2. Monolayer adsorption of B6 anti-bml CTL: (A) adsorbed to B10.D2 (H-2^d) monolayer; (B) adsorbed to dm2 (H-2^dm2) monolayer; (C) adsorbed to B10.BR (H-2^k) monolayer; (D) adsorbed to C3H.OH (d/k) monolayer, and (E) adsorbed to B10.A(4R) (k/b) monolayer. In parentheses, the H-2 haplotype or H-2K and -D alleles.
Discussion

B6 anti-bm1 CTL are exclusively generated against novel H-2K antigens created by the following three AA substitutions: Glu → Ala at position 152, Arg → Tyr at position 155, and Leu → Tyr at position 156 (8, 9). The L^d molecule shares the AA at these positions with K^bm1 in addition to all other AA from positions 146–162 (8–11). If primary AA sequences are the target structure for alloimmune CTL, the H-2L^d molecule, on the basis of its structural identity at positions 146–162 with bm1, should bear all target structures recognized by B6 anti-bm1 CTL. However, both in direct lysis experiments and monolayer adsorption experiments this is clearly not the case. Neither the adsorption onto H-2L^d-bearing monolayers nor the adsorption onto monolayers of other H-2 types substantially reduced the CTL activity against bm1 target cells. Therefore, the presence of the 146–162 AA sequence in the K^bm1 molecule creates a unique bm1 target determinant absent from L^d. In addition, B6 anti-bm1 CTL were shown to include separate subsets reactive with K^k, D^k, and H-2^d minus L^d, none of which could be adsorbed onto L^d-bearing monolayers. Therefore, B6 anti-bm1 CTL contain at least four CTL subsets not reactive with L^d (Table I).

These findings can only be explained by assuming that the presence of the 146–162 AA sequence in the K^bm1 molecule creates conformational determinants different from those induced by the same AA sequence in the context of the L^d molecule.

This conclusion is further supported by the earlier observation that B6 anti-bm1 CTL cross-reactive with several other K^b mutants that do not share any primary structural homology with the mutated portion of the K^bm1 molecule (7). Apparently, these mutations in different parts of the H-2 K^b molecule result in similar new conformational determinants (4, 7). The identity of the AA sequence of the K^bm1 molecule from positions 146–162 with the L^d molecule, raised doubts on point mutation as the mechanism underlying the generation of H-2 mutants. As an alternative, gene conversion was proposed to explain this finding. Gene conversion is a genetic event in which a particular gene segment is transferred from one homologous gene to another (8, 9). The sharing of an AA segment between K^bm1 and L^d on the basis of gene conversion can be explained in two ways. The bm1 mutation originated in a (B6 × BALB/c)F_1 mouse where gene conversion could have occurred, or, the B6 genome contains an Ld-like pseudo-gene which by definition remains normally silent. In favor of gene conversion is the finding that identical complex mutations occurred repeatedly and independently of each other and that many mutations show clusters of AA changes.

| Subset | Specificity | Relevant adsorption (Fig.) | Reactive with L^d |
|--------|-------------|-----------------------------|-------------------|
| 1      | bm1 unique  | 2, A–E                      | –                 |
| 2      | K^k         | 2 D                         | –                 |
| 3      | D^k         | 2 E                         | –                 |
| 4      | H-2^d minus L^d | 2 A and B | –                 |
| 5^2    | D^k         | 2 E                         | ?                 |
| 6      | L^d         | 2 B                         | +                 |

Table I: Distinct CTL Subpopulations Among B6 Anti-bm1 CTL
requiring multiple base substitutions (8, 9, 13). Gene conversion has also been invoked to explain the differences among HLA-B7, HLA-28, and HLA-A2 in the first variable segment (14).

Our data also correspond with those of Hunt and Sears (15), whose studies indicated that structural homology between two class I molecules is not necessarily associated with CTL cross-reactivity. For example, in their study b-anti-bml CTL cross-reacted only partially with Ld-positive target cells of the H-2a haplotype, in agreement with our data.

With regard to the question whether MHC-restricted CTL also recognize conformational determinants, it is striking that bml CTL specific for lymphocytic choriomeningitis (LCM) virus, vaccinia virus, and ectromelia virus do not recognize virus-infected target cells expressing H-2Ld (16, 17). Moreover, the bml mutant has gained new restriction specificities unique for Kbml in the TNP-specific CTL response (de Waal et al., unpublished observations), whereas H-2Ld-restricted TNP-specific CTL responses were not observed (18). Conversely, in H-2d mice the vesicular stomatitis virus (VSV)-specific CTL response is solely restricted by H-2Ld (19), whereas bml is a CTL nonresponder against VSV (J. Forman, personal communication). These findings can again be explained best by assuming that the 146–162 segment in the Kbml molecule creates conformational determinants different from those in H-2Ld.

Taken together, our data strongly strengthen the notion that CTL recognize conformational determinants and not primary amino acid sequences. Further insight into the three dimensional structure of MHC antigens is needed to answer the question of what T cells really see.

Summary

The bml H-2Kb mutant differs from the parental strain C57BL/6 (B6) only at amino acid (AA) positions 152, 155, and 156 of the H-2K molecule. The H-2Ld molecule is structurally identical with the H-2 Kbml molecule from positions 146–162, thus including all three AA substitutions in Kbml. In direct lysis and monolayer adsorption studies, B6 anti-bml cytotoxic T lymphocytes (CTL) were shown to include at least five distinct CTL subsets of the following specificities. (a) Uniquely reactive with Kbml; (b) cross-reactive with Kd; (c) cross-reactive with Dd; (d) cross-reactive with H-2d minus Ld, and (e) cross-reactive with Ld. If B6 anti-bml CTL were directed against the primary AA-sequence difference, then all five subsets are expected to react with Ld. However, four out of five CTL subsets including a major population uniquely directed against Kbml failed to react with Ld.

These findings strongly strengthen the notion that CTL recognize conformational determinants and not primary AA sequences.

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