The Journal of Immunology

RESEARCH ARTICLE | JULY 01 1980

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J Immunol (1980) 125 (1): 74–77.
https://doi.org/10.4049/jimmunol.125.1.74

0022-1767/80/1251-0074$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 125, No. 1, July 1980
Printed in U.S.A.

MULTIPLE CTL SUBSETS GENERATED BY H-2.L LOCUS PRODUCTS STIMULATION: EVIDENCE FOR AN ANTIGENIC DETERMINANT PRIVATE TO H-2.L^{d} [1]

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Analysis of the fine specificity of CTL subpopulations raised by an H-2.L locus products stimulation (H-2^{dm2} anti-H-2^{d}) was performed by absorption experiments by using monolayers of macrophages of H-2^{m}, H-2^{q}, H-2^{b}, and H-2^{k} haplotypes. The results show the existence of four CTL subsets. The pattern of reactivity of three of them could be correlated with that of antibodies present in H-2^{dm2} anti-H-2^{d} antisera (anti-H-2.64, anti-H-2.65, and anti-H-2.K^{k}). The fourth CTL subset reacted with a specificity unique to H-2.L^{d} molecules (a private specificity?), absent on cells from H-2^{m}, H-2^{q}, H-2^{b}, and H-2^{k} haplotypes, and undescribed as yet by serologic methods. These data support the hypothesis that the H-2.L locus products are comparable in their antigenic properties to those of the H-2.K and H-2.D loci.

By using antisera reacting with public specificities of the H-2.28 family (1), a new D region-coded H-2 molecule, different from the classical H-2.D molecule bearing the D region private specificity, has been described by co-capping studies (2, 3). This molecule, further named H-2.L, has a m.w. of 45,000 and precipitates, on soluble extracts, independently from H-2.D (4, 5). Additional evidence for separate H-2.D and H-2.L molecules came from the finding that the D region H-2 mutant of loss type, BALB/c-H-2^{dm2}, when immunized with parental H-2^{d} cells, develops antisera that have the pattern of reactivity of some of the anti-H-2.28 antisera (6). The definitive demonstration of the absence of H-2.L molecules in cells of the H-2^{dm2} mutant came from Hansen *et al.* (7) showing that no 45,000-dalton peak could be obtained after immunoprecipitation of H-2.K and H-2.D molecules.

In view of the absence of recombinants between H-2.D and H-2.L regions (8), the serologic definition of the H-2.L molecule is not clarified as yet. Besides the fact that the H-2.L molecule reacts with antibodies present in all anti-H-2.28 antisera (see above), the use of H-2^{dm2} anti-H-2^{d} antisera led to the description of several public specificities on H-2.L products of H-2^{d}, H-2^{q} haplotypes (H-2.64,65) and of H-2^{b} (H-2.64) (9). More recently (10, 11), such antisera, taken during the course of immunization of individual mice, were found to react also with H-2^{p}, H-2^{r}, and H-2^{k} haplotypes (in the H-2^{k} haplotype, the reaction was located on H-2.K molecules). However, in contrast to H-2.K and H-2.D molecules, no private specificity has yet been defined on the H-2.L molecule, and the H-2.L^{d} and H-2.L^{q} molecules are considered to be serologically similar.

Like the H-2.K and H-2.D molecules, the H-2.L molecules bear not only serologically detectable determinants but also determinants that behave as targets for cytotoxic T lymphocytes (CTL)[2] developed either in semi-alloreactive combinations (12, 13) or in an H-2^{dm2} anti-H-2^{d} stimulation (12, 14, 15). Recently, analyzing the alloreactive cytotoxic T cell response, we have shown that different subpopulations of CTL are raised against the H-2 specificities (private or public) expressed on one H-2 molecule (13). In the work presented herein, using absorption of CTL on macrophage monolayers, we analyzed the fine specificity of CTL generated by an H-2^{dm2} anti-H-2^{d} stimulation. The results suggest that in this situation at least four different subpopulations of CTL can be generated. The pattern of reactivity of three of them could be correlated to that of antibodies present in H-2^{dm2} anti-H-2^{d} antisera. Interestingly, the fourth subpopulation reacted against a determinant specific to the H-2.L^{d} molecule (a private specificity?) and absent on cells of H-2^{q} and H-2^{b} haplotypes.

MATERIALS AND METHODS

Mice. Two- to 3-month-old mice of both sexes listed in the tables were kindly provided by Prs. J. Colombani and J. P. Levy (SNC7, INSERM, Paris). H-2^{dm2} mutant mice were a gift of Dr. P. Demant (Netherlands Cancer Institute, Amsterdam).

Source of alloreactive CTL. Spleen cells were obtained from mice once primed *in vivo* with an i.p. injection of 5×10^{7} allogeneic spleen cells, then restimulated 11 days later *in vitro* with the same stimulator cells. The mixed lymphocyte cultures (MLC) consisted of 5×10^{7} mitomycin-treated stimulator cells (50 μg/ml of mitomycin for 30 min) mixed with 1×10^{8} responder cells in 50 ml of culture medium. Culture medium was RPMI 1640 (Eurobio, France) supplemented with 5% fetal calf serum (Eurobio), antibiotics, and 5×10^{-5} M β-mercaptoethanol. The cultures were incubated at 37°C during 5 days in an humidified atmosphere containing 5% CO_2 in air.

Absorption of alloreactive CTL on macrophage monolayers. The procedure was based on that originally described by Brondz and Snegirova (16) and was modified according to the method of Kees *et al.* (17). Mouse peritoneal macrophages were harvested 2 days after i.p. injection of 2 ml of thioglycollate medium (Difco Laboratories, Detroit, Mich.). 2×10^{7} macrophages in 5 ml medium were seeded in 25 cm^{3} tissue culture Falcon flasks

Received for publication January 29, 1980.
Accepted for publication March 18, 1980.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by grants from the Institut National de la Santé et de la Recherche Medicale (N° 78-4-1401), from the Délégation Générale à la Recherche Scientifique et Technique (N° 79-7-275) and from the Centre National de la Recherche Scientifique (ATP "Immunologie"-1979).

[2] Abbreviations used in this paper: CTL, cytotoxic T lymphocytes.

(3013) in order to obtain uniform cell monolayer and were incubated for 24 to 48 hr at 37°C. Two milliliters of culture medium containing 10^7 alloimmune spleen cells were then placed on the prewashed monolayers. Absorption was carried out for 90 min at 37°C and was followed by the recovery of the nonadherent cells. Less than 1% detached macrophages were contaminating these cells, as seen by morphologic criteria, which rules out the possibility that detached cells may compete with target cells during the subsequent cytotoxic assay.

Cytotoxic assay. It was done according to the method of Cerottini *et al.* (18). Target cells were PHA-stimulated spleen cells (1 μg/ml of PHA, Wellcome Laboratories, England). 1×10^7 blast cells were labeled with 200 μCi of ^{51}Cr (CEA, France) for 90 min at 37°C. After washings, 2×10^4 target cells were mixed in microtest plates (Falcon Tc 3040) with 1×10^6 effector cells in 200 μl of culture medium. Duration of the assay was 3½ hr, and percent-specific ^{51}Cr release was calculated as:

$$\frac{E - C}{T - C} \times 100,$$

where E is the release in the presence of immune spleen cells, C the release in the presence of unstimulated spleen cells, and T the maximum release upon the addition of HCl 4N. Spontaneous release did not exceed 20 to 30% of the total release.

RESULTS

Analysis of H-2^{dm2} anti H-2^d cross-reactivities exerted by CTL. In the first set of experiments, CTL generated in the H-2^{dm2} anti-H-2^d (BALB/cKh) combination were tested on a panel of target cells from various haplotypes. In accordance with other reports (14, 15) a strong T cell-mediated cytotoxicity (more than 70% specific lysis) was detected against H-2.L^d gene products of the BALB/cKh target cells (Table I). This cytotoxicity was completely inhibited by an H-2^{dm2} anti-H-2^d antiserum (91 ± 2.3% inhibition of lysis) but not by antibodies directed against the H-2.K or H-2.D gene products in which the inhibition was identical to that exerted by normal mouse serum (20%), which is in agreement with the fact that H-2.L molecules are expressed on the cell surface independently from H-2.K and H-2.D molecules (4, 5). When the cytotoxic activity of these CTL was tested on a panel of target cells of different haplotypes, different levels of cross-killing were observed.

B10.AKM (H-2^m:K^k D^q L^q) and C3H.Q (H-2^q) targets were killed at a high extent (40% of lysis), B10 (H-2^b), B10.BR (H-2^k), C3H.NB (H-2^p), and B10.RIII (H-2^r) at a lower extent (between 25 and 13% of lysis), and B10.S (H-2^s) targets were not killed. The almost negative killing of C3H.OL (K^d D^k L^k) (7.3%) compared with B10.BR (H-2^k) (20,5%) suggests that the cross-reaction with B10.BR is located mostly on the H-2.K molecule.

Interestingly, the H-2^{dm2} anti-H-2^d response, revealed at the cytotoxic T cell-mediated level, follows grossly the pattern of reactivity of H-2^{dm2} antisera (8–11). However, it was clear from the present data that BALB/c (H-2^d) targets were killed to a much higher extent than B10.AKM (K^k D^q L^q) or C3H.Q (H-2^q) (71% compared with 40%).

This finding was unexpected according to H-2 serology, since H-2.L molecules of H-2^d and H-2^q haplotypes are considered to be serologically similar (both express H-2.64 and H-2.65, and antibodies present in H-2^{dm2} anti-H-2^d antisera are completely absorbed by H-2^q) (9).

Therefore, in the next step of our work, absorption experiments were designed in order to see whether CTL could detect a specificity peculiar to H-2^d and absent on H-2^m and other haplotypes.

TABLE I

Cross-killing exerted by H-2^{dm2} anti-BALB/cKh CTL (anti H-2.L^d) on targets of different strains

Targets	% of Lysis[a]	10% 20% 30% 40% 50% 60% 70%
H-2^{dm2} (K^d D^d Lθ^b)	2.3 ± 1.2	
BALB/cKh (K^d D^d L^d)	71.5 ± 2.1	
B10.AKM (K^d D^q L^q)	41.4 ± 1.2	
C3H.Q (K^q D^q L^q)	40.5 ± 1.9	
B10 (K^b D^b L^b)	25.3 ± 0.8	
B10.BR (K^k D^k L^k)	20.6 ± 1.8	
C3H.NB (K^p D^p L^p)	18.2 ± 2.1	
B10.RIII (K^r D^r L^r)	13.8 ± 1.3	
C3H.OL (K^d D^k L^k)	7.5 ± 0.9	
B10.S (K^s D^s L^s)	2.4 ± 1.3	

[a] Mean values of triplicates ± S.D.

[b] Alleles at the H-2.K, H-2.D, and H-2.L regions. θ, absence of H-2.L molecules.

Evidence for different CTL subsets raised against the H-2.L molecule. H-2^{dm2} anti-H-2^d CTL were absorbed on macrophages from different strains, and the residual cytotoxic activity of the nonadherent population was tested on a panel of cross-reacting target cells. The results are shown in Table II.

First, absorption of the CTL on H-2^{dm2} macrophages did not significantly reduce the degree of cytotoxicity directed against the various targets, whereas their absorption of H-2^d macrophages removed up to 85% of their cytotoxic potential on all targets tested, showing the specificity and the efficiency of the absorption procedure.

Absorption on B10.AKM (K^k D^q L^q) monolayers, which in serological terms are supposed to be identical to BALB/cKh, left a subpopulation still able to kill H-2^d targets at a high extent (43% of lysis). This subpopulation did not kill C3H.Q (H-2^q) targets (identical in the H-2.D and H-2.L loci with B10.AKM), B10.BR (H-2^k) (identical in the H-2.K locus with B10.AKM), and B10 (H-2^b) (which expresses some but not all the specificities of H-2.L^d and H-2.L^q molecules). Therefore, these results suggest the existence on the H-2.L molecule of determinants specific to H-2^d and different from the determinants described so far by serologic methods.

In conclusion, these data provide evidence for the existence on the H-2.L molecule of a determinant specific to H-2^d (a private specificity?) and revealed a subpopulation of CTL different from those recognizing the cross-reacting determinants (public specificities) expressed on the same molecule.

TABLE II

Specific adsorption of H-2^{dm2} anti-BALB/cKh CTL on macrophage monolayers[a]

Adsorption Monolayers	Target Cells					
	H-2^{dm2} K^d D^d Lθ^b	BALB/cKh K^d D^d L^d	B10.AKM K^k D^q L^q	C3H.Q K^q D^q L^q	B10 K^b B^b L^b	B10.BR K^k D^k L^k
NO	2.3 ± 1.2	71.5 ± 2.1	41.4 ± 1.2	40.5 ± 1.9	25.3 ± 0.8	20.6 ± 1.8
H-2^{dm2}	2.5 ± 0.5	64 ± 1.9	37.9 ± 1.3	37.6 ± 1.1	22.1 ± 0.4	18.3 ± 0.9
BALB/cKh	2.4 ± 0.9	10.1 ± 0.9	5.6 ± 1.5	4.6 ± 1.1	3.8 ± 1.3	4 ± 0.8
B10.AKM	1.1 ± 1.1	[c]43.6 ± 1.4	4.1 ± 0.9	0.5 ± 1.2	4.2 ± 2.5	2.9 ± 1.6
C3H.Q	2.2 ± 1.3	[c]46.5 ± 2.4	[c]16.9 ± 1.5	5.2 ± 1.1	4 ± 1	[c]14.1 ± 1.7
B10.BR	1.3 ± 0.8	[c]56.5 ± 1.5	[c]28.6 ± 0.7	[c]34.1 ± 1.6	[c]20.1 ± 0.6	3.7 ± 1.8
B10	0.9 ± 0.4	[c]55.6 ± 2.6	[c]24.6 ± 1.2	[c]19.7 ± 1.5	2.7 ± 0.7	[c]16.7 ± 2.1

[a] Data are the mean values of triplicates ± S.D.

[b] Alleles at the H-2.K, H-2.D and H-2.L regions. θ, absence of H-2.L molecules.

[c] Significantly more than when tested on target cells syngeneic with macrophage monolayers.

In order to further analyze the CTL subpopulations raised against the H-2.L molecule, absorption on macrophages from other haplotypes were performed. As shown in Table II, complete absorption of CTL with C3H.Q (H-2^q) monolayers left a subpopulation still able to lyse B10.BR (H-2^k) (14% of lysis) and B10.AKM (K^k D^q L^q) (17% of lysis) targets, but ineffective on B10 (H-2^b), suggesting the existence of a subpopulation cross-reacting with H-2.K^k molecules but different from that cross-reacting with H-2^q and H-2^b. Indeed, absorption of CTL on B10.BR left a residual cytotoxicity on B10.AKM (28% of lysis), C3H.Q (34% of lysis), and B10 (20% of lysis), which corresponds to CTL cross-reacting with H-2^q and H-2^b but not with H-2.K^k. Therefore, these absorption procedures suggest the existence of three independent subpopulations of CTL generated by an H-2.L locus stimulation: a first one recognizing a determinant specific to H-2.L^d, a second cross-reacting with H-2.K^k, and a third cross-reacting with H-2^q and H-2^b.

A more precise determination of CTL cross-reacting with H-2^q and H-2^b was achieved by absorbing the CTL on B10 monolayers. In fact, this absorption left a subpopulation that still killed 20% of C3H.Q targets (by contrast, as shown above, absorption on C3H.Q removed the cytotoxicity directed against B10). Moreover, B10.BR targets were also killed by this subpopulation, confirming that the CTL that cross-react with H-2^b are different from those that cross-react with H-2.K^k. Therefore, the CTL cross-reacting with H-2^q consist of at least two subsets: one that recognizes determinants specific to H-2^q but absent on H-2^b, and one recognizing determinants shared by H-2^q and H-2^b.

DISCUSSION

In the present report, the fine specificity of CTL raised against H-2.L antigens was investigated. First, their pattern of reactivity on a panel of target cells followed grossly that of H-2^{dm2} anti-H-2^d antisera described by several authors. Second, the H-2.L molecule was found to be recognized by at least four independent subpopulations of CTL: one reacting against a determinant specific to H-2.L^d and absent on H-2^m, H-2^q, or H-2^b; a second cross-reacting with the H-2.K^k molecule; a third cross-reacting with H-2^q but not H-2^b; and a fourth cross-reacting with H-2^q and H-2^b.

Interestingly, the pattern of reactivity exerted by H-2^{dm2} anti-H-2^d CTL and H-2^{dm2} anti-H-2^d antisera are very similar, since both give positive reactions with cells of H-2^q, H-2^m, H-2^b, H-2^p, H-2^r and H-2^k haplotypes (8–11). In this last haplotype, the cross-reactivity exerted by CTL was found to be located on the H-2.K molecule, like that exerted by H-2^{dm2} anti-H-2^d antisera (10, 11). In addition, B10.S (H-2^s) targets, which react weakly (10) or not at all (8, 9) with H-2^{dm2} anti-H-2^d antisera, were not killed by the CTL. These observations confirm and extend previous findings (13, 19, 20), showing that in an alloreactive response the cross-reactivities exerted by the CTL appear to be directed mostly against the public specificities of the H-2 molecules.

The absorption studies, showing the existence of a subpopulation of anti-H-2.L CTL recognizing determinants present on H-2^q (C3H.Q) but absent on H-2^b (B10) and another recognizing determinants common to H-2^q and H-2^b, are also in strict correlation with the analysis of antibodies present in H-2^{dm2} anti-H-2^d antisera. As shown previously (9), the reactivity of this antiserum on H-2^b could be completely absorbed by H-2^q but not vice versa, demonstrating the existence of a specificity common to H-2.L molecules of H-2^b and H-2^q (H-2.64) and a specificity expressed on H-2^q but not on H-2^b (H-2.65). More recently (8), the analysis of reactivity of H-2^{dm2} anti-H-2^d antisera on cells from congenic wild mice led to the description of several new specificities on the H-2.L^q molecules (H-2.73, 74, 75), most of them absent on H-2.L^b, data that are still in agreement with the present CTL analysis.

The existence of a separate CTL subset cross-reacting with the H-2.K^k molecule is also compatible with the serologic cross-reactivity of H-2^{dm2} anti-H-2^d antisera (10, 11), although the presence of anti-K^k antibodies in such antisera may reflect a response to virus modified H-2^d components rather than public specificities shared by H-2.L^d and H-2.K^k molecules (21).

In contrast to the observation that all the cross-reactions at the T cell level appeared to correspond to those observed with antibodies, the absorption of CTL on B10.AKM (K^k D^q L^q), which theoretically should remove all the CTL reacting with BALB/c, left a high level of cytotoxicity on BALB/c. One could argue that these results are due to a lower expression of H-2.L molecules on B10.AKM compared with BALB/c. This does not seem to be the case, since increasing the amount of macrophages used for absorption did not modify the residual cytotoxicity on BALB/c. In view of 1) the mouse strains used to prepare CTL (H-2^{dm2} and H-2^d are identical in their T region loci), and 2) the fact that the killing of BALB/cKh targets was completely inhibited by anti-H-2.L but not anti-H-2.K or anti-H-2.D antisera (data not shown, and 12, 15), it appears unlikely that the nonadherent CTL subset reacts on H-2^d with other molecules than H-2.L. Since this subset did not kill target cells from other haplotypes than H-2^d (H-2^m, H-2^q, H-2^b and H-2^k), it seems rather to react with a specificity unique to the H-2.L molecules of H-2^d haplotype. This specificity might either correspond to the private specificity of the H-2.L^d molecule, as yet not discovered by the serologic methods, or to an antigenic determinant which, as in other mutant-parent combinations, does not induce antibody production (22). Interestingly, the existence of a spec-

ificity present on the H-2.L molecule of H-2^d but absent on H-2^q appears in correlation with the recent peptide map analysis of these molecules showing that H-2.L^d and H-2.L^q molecules are different (23).

The fact that an allosensitization against the H-2.L molecule generates at least four separate CTL subsets is in accordance with our previous data showing that the private and the cross-reacting (public) determinants of the H-2.K^d molecule are recognized by at least two different subpopulations of CTL (13). They confirm also results of Geib *et al.* (24), which showed, by using mutants at the H-2.K^b locus, that multiple CTL subsets are raised against the H-2.K molecule.

The data presented here provide evidence for the polymorphism of the H-2.L locus revealed at the T cell-mediated level and for the existence of specificity unique to the H-2.L molecule of H-2^d (a private specificity?). These findings extend the previous observations that in their serologic and biochemical (25) properties the products of the H-2.L locus are comparable to those of the H-2.K and H-2.D loci.

Acknowledgments. We wish to thank Dr. W. H. Fridman for very helpful discussions, Dr. J. P. Levy, Dr. P. Demant, and Pr. J. Colombani for providing H-2^{dm2} mutant and congenic mice.

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