ROLE OF *H-2* LYMPHOCYTE-DEFINED AND SEROLOGICALLY-DEFINED COMPONENTS IN THE GENERATION OF CYTOTOXIC LYMPHOCYTES*

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The major targets for cytotoxic lymphocytes (CL's) in mouse are determined by genes of the *H-2* complex. These *H-2* loci, alleles of which are important in determining histocompatibility antigens, can be divided into two groups, genetically and functionally distinct. The Hld (histocompatibility LD) loci differences lead to strong lymphocyte proliferative responses in MLC and graft-vs.-host (GvH) reactions; Hsd (histocompatibility SD) loci (*H-2K* and *H-2D*) differences usually lead to a relatively weak MLC or GvH response (1–5). Lymphocytes activated in MLC against allogeneic cells differing by both *H-2* lymphocyte-defined (LD) and serologically-defined (SD) components develop cytotoxic potential against target cells carrying those same foreign antigens. This cytotoxic test is referred to as cell-mediated lympholysis (CML). Whereas LD differences are of prime importance in leading to a lymphocyte proliferative response in MLC, the SD antigens, or the phenotypic product of genes very closely linked to the SD alleles, serve as the best targets in CML (6–8). We shall refer to these targets as SD antigens.

While LD differences on the sensitizing (stimulating) cells in MLC facilitate the development of cytotoxic reactions against the SD antigens, these determinants alone lead to only weak cytotoxicity in CML (7, 8, footnote 1). At least two possibilities can be offered to explain this observation: (*a*) LD differences alone in MLC do lead to generation of cytotoxic lymphocytes but these LD differences do not serve as adequate targets in CML; (*b*) proliferation due to LD differences alone does not lead to the formation of effective CL's; the SD antigenic differences during the MLC phase facilitate the development of strong cytotoxic activity and act also as the specific target in CML.

The mechanisms by which sensitized thymus-derived (T) lymphocytes mediate cytotoxicity against target cells remains largely obscure. The present day concept suggests that specificity of cytotoxic effect is due to receptors on the surface of the T-effector lymphocytes which recognize target cell antigens and thereby allow specific cell-cell contact. Once the target and effector cells are in proximity, target cell destruction is mediated nonspecifically. Forman and Möller recently described such nonspecific target cell destruction (9). They demonstrated that lymphocytes sensitized to alloantigens in MLC are cytotoxic not only to target cells

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carrying those same antigens, but even to isogeneic (self) targets when phytohemagglutinin (PHA) is added to the mixture of sensitized cells and target cells during the cytotoxicity assay (PHA-dependent cytotoxicity). To the extent that PHA-dependent cytotoxicity can be used as a model for assessing the presence of CL's in CML, we have used this system to determine whether proliferative responses to LD antigens alone can "generate" cytotoxic cells. Furthermore we have used, as target cells, normal lymphocytes which bear LD antigens to ascertain whether the LD products on these cells can serve as targets in CML.

Materials and Methods

Mice used in these studies were raised in our own colony; breeding pairs were obtained from J. Stimpfling, J. Klein, and Jackson Laboratories, Bar Harbor, Maine. *H-2* differences in the strains used have been discussed previously (1, 8, 10, 11).

A modification of the MLC technique of Widmer (12) and Phillips (13) is used to generate cytotoxic effector lymphocytes. Briefly, 40×10^6 responding spleen cells and 40×10^6 mitomycin C-treated stimulating spleen cells are suspended in 20 ml of RPMI-1640 containing 5% inactivated (56°C for 30 min) human pooled plasma and cultured in Falcon tissue culture flasks (no. 3012, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) (in a vertical position) in a humidified atmosphere of 5% CO_2 in air. After 5 days of incubation MLC activation is measured by tritiated thymidine uptake and cell-mediated lympholysis on sodium chromate (^{51}Cr)-labeled target cells as previously described (7). Lymphocytes used for target cells are either (*a*) PHA-stimulated lymph node cells (14) or (*b*) fresh lymph node cells, i.e., lymph nodes removed from normal animals on the day of CML assay. PHA-dependent cytotoxicity differs from the above in that PHA-M (Difco Laboratories, Detroit, Mich.) is added to a final concentration of 250 μg/ml to the mixture of effector and target cells during the 3 h incubation for the assay of lympholysis; this should not be confused with the normal CML assay in which target cells are in some cases stimulated with PHA for 3 days before labeling with ^{51}Cr.

Results

Results of two of the six experiments performed are shown in Tables I and II. In Table I are given results with PHA-stimulated blast cells as target cells; in Table II, fresh lymphocytes are used. In each table MLC results are given using cells from strains differing by *H-2* LD and SD factors and MLC mixtures differing by LD factors alone. The cytotoxic assay is carried out either as in normal CML tests or in the presence of PHA (columns 1 and 2, respectively, under "percent cytotoxicity").

Cultures activated with cells which differ for both LD and SD components generate CL's active against the specific target. In spite of significant and similar MLC activation, combinations which differ only by LD show little if any lysis of specific "targets" [AQR or B10.T(6R)] or of unrelated targets bearing LD and SD differences from the sensitizing cells in CML.

The addition of PHA to the cultures during the 3 h incubation to determine PHA dependent cytotoxicity has different effects depending on the genetic differences existing during the MLC activation phase in the experiment: LD + SD-activated cultures generate greater cytotoxic activity against target cells syngeneic to the effector cell than when the MLC is activated by LD differences alone, despite equivalent levels of proliferative response. Cultures activated by LD only showed

TABLE I
PHA-Dependent Cytotoxicity with PHA-Blast Lymphocytes as Targets

MLC*	Sensitization of effectors‡	Target	% Cytotoxicity ± SD‖	
			No PHA added during CML assay	PHA added during CML assay
	LD + SD			
19,819 ± 439	B10.T (6R) + C57BL/10_m§	AQR	2.3 ± 2.4	36.3 ± 3.6
		B10.T (6R)	−8.0 ± 1.7	31.8 ± 4.7
		C57BL/10	68.2 ± 4.1	77.9 ± 3.5
13,303 ± 1,075	AQR + C57BL/10_m	AQR	0.6 ± 0.8	40.1 ± 0.9
		B10.T(6R)	−7.1 ± 1.7	36.8 ± 2.6
		C57BL/10	71.8 ± 4.4	68.9 ± 4.0
	LD			
12,205 ± 604	B10.T(6R) + AQR_m	AQR	−4.4 ± 1.6	1.7 ± 1.2
		B10.T(6R)	−14.7 ± 1.7	−3.1 ± 1.7
		C57BL/10	−11.4 ± 3.6	−1.6 ± 5.4
14,771 ± 756	AQR + B10.T$(6R)_m$	AQR	−4.9 ± 1.8	5.4 ± 2.4
		B10.T(6R)	−9.5 ± 1.7	2.8 ± 2.5
		C57BL/10	−11.2 ± 3.8	1.4 ± 5.1

* Mean cpm of triplicate values ± SD; control values are B10.T(6R) + B10.T$(6R)_m$ = 761 ± 86; AQR + AQR_m = 1,577 ± 47.

‡ We refer to the four regions of the MHC as K, I, S, D. The loci, alleles of which determine the SD antigens are in the K and D regions; the strong LD locus appears to be in the I region. AQR = qkdd; B10.T(6R) = qqqd; C57BL/10 = bbbb.

§ Reciprocal combinations (not shown) were also done with a comparable pattern of results.

‖ Percent cytotoxicity is calculated

$$\frac{ER - SSR}{MR - SSR} \times 100$$

where experimental release (ER) represents mean counts per minute (cpm) released from ^{51}Cr labeled target cells incubated with effectors sensitized to either LD or LD + SD differences; specific spontaneous release (SSR) is the mean cpm released from the target when incubated with cultured cells syngeneic to the target, e.g. AQR + AQR_m/AQR target; maximum release (MR) represents the mean cpm released from target cells after rapid freeze-thaw treatment. Target AQR, MR = 2063 ± 5.6, SSR = 570 ± 9.5, SSR (PHA added during CML assay) = 355 ± 13.4 Target B10.T(6R), MR = 2,967 ± 162.3, SSR = 818 ± 21.0, SSR (PHA added during CML assay) = 571 ± 28.5 Target C57BL/10, MR = 2,582 ± 66.0, SSR = 823 ± 52.5, SSR (PHA added during CML assay) = 549 ± 104.9.

only a marginal increase in percent cytotoxicity in the presence of added PHA in all experiments except one, in which the addition of PHA during the cytotoxicity assay led to significant levels of lysis. In that one experiment, the percent lysis in PHA-dependent cytotoxicity after activation by LD and SD differences remained significantly higher in every case (range 22.2%–55.7%) than after activation by LD differences alone (range 8.1%–29.8%). (Mice used in this experiment had been vaccinated for ectromelia due to an outbreak in another colony on our campus; whether this is related to the enhanced PHA-dependent cytotoxicity seen is not known.)

TABLE II
PHA-Dependent Cytotoxicity with Fresh Lymphocytes as Targets

MLC*	Sensitization of effectors‡	Target	% Cytotoxicity ± SD‖	
			No PHA added during CML assay	PHA added during CML assay
	LD + SD			
16,145 ± 826	B10.T (6R) + C57BL/10_m§	AQR	2.1 ± 4.6	37.0 ± 5.7
		B10.T (6R)	−1.0 ± 4.5	28.4 ± 4.4
		C57BL/10	55.4 ± 5.0	70.5 ± 2.8
9,391 ± 433	AQR + C57BL/10_m	AQR	5.3 ± 3.3	40.0 ± 5.1
		B10.T (6R)	3.4 ± 4.4	37.1 ± 2.9
		C57BL/10	52.0 ± 7.1	59.2 ± 5.9
	LD			
10,097 ± 274	B10.T (6R) + AQR_m	AQR	1.4 ± 4.7	10.8 ± 6.7
		B10.T (6R)	−4.4 ± 5.6	5.0 ± 3.9
		C57BL/10	3.1 ± 6.2	7.6 ± 5.5
8,253 ± 317	AQR + B10.T $(6R)_m$	AQR	0.4 ± 4.6	5.8 ± 4.7
		B10.T (6R)	8.7 ± 4.1	12.4 ± 3.0
		C57BL/10	3.7 ± 4.5	12.6 ± 5.6

* Mean cpm of triplicate values ± SD; control values are B10.T (6R) + B10.T $(6R)_m$ = 1,128 ± 88; AQR + AQR_m = 2,223 ± 64.

‡ We refer to the four regions of the MHC as K, I, S, D. The loci, alleles of which determine the SD antigens, are in the K and D regions; the strong LD locus appears to be in the I region. AQR = qkdd; B10.T (6R) = qqqd; C57BL/10 = bbbb.

§ Reciprocal combinations (not shown) were also done with a comparable pattern of results.

‖ Percent cytotoxicity is calculated

$$\frac{ER - SSR}{MR - SSR} \times 100$$

where experimental release (ER) represents mean cpm released from ^{51}Cr labeled target cells incubated with effectors sensitized to either LD or LD + SD differences; specific spontaneous release (SSR) is the mean cpm released from the target when incubated with cultured cells syngeneic to the target, e.g. AQR + AQR_m/AQR target; maximum release (MR) represents the mean cpm released from target cells after rapid freeze-thaw treatment. Target AQR, MR = 664 ± 27.1, SSR = 212 ± 10.1, SSR (PHA added during CML assay) = 215 ± 19.0 Target B10.T (6R), MR = 689 ± 18.5, SSR = 239 ± 18.3, SSR (PHA added during CML assay) = 244 ± 11.0 Target C57BL/10, MR = 712 ± 314, SSR = 239 ± 14.0, SSR (PHA added during CML assay) = 233 ± 24.1.

These data further demonstrate that if both SD and LD differences are present during the MLC sensitization phase, the effector cells generated are cytotoxic not only to specific target cells in CML but also to isogeneic and other target cells in PHA-dependent cytotoxicity. However, with only LD differences present during the MLC sensitization procedure (identity for the SD antigens) despite an approximately equivalent proliferative response in MLC, the cells are only minimally cytotoxic to any target cells. This is true both with target cells stimulated 3 days previously with PHA and with fresh target cells. To the extent that PHA-dependent cytotoxicity can be used to argue about CL formation in general these re-

sults suggest strongly that SD differences present during MLC sensitization lead to the effective generation of CL's.

Discussion

We have proposed that the LD-SD collaboration in CML may be explained by the existence of two populations of lymphocytes: one responsive to LD differences and the other to SD differences. The cell population responding to the SD differences would function as the cytotoxic lymphocytes in CML (15). If this model is correct, one might imagine that the SD responsive cells are also the most active in the PHA-dependent cytotoxicity—thus the greater cytotoxicity even on isogeneic target cells given an SD difference in the original sensitizing MLC mixture. If some proportion of the LD responding cells also respond to SD antigens on a given allogeneic stimulating cell, i.e., one cell can respond to both LD and SD differences, one would have to conclude that the SD stimulus is in some way helpful in promoting differentiation of these cells to cytotoxic lymphocytes.

We have recently suggested that *H-2* LD differences may be able to lead to positive CML (8, footnote 1) even though they are not as "strong" in this regard as are the SD differences. This would provide further evidence that the differences between *H-2* LD and SD components are, at least in some respects, of a quantitative nature, as we have suggested in the past (8, 16, 17). It would appear to us that the seeming relative importance of LD components in the proliferative phase of the MLC test and SD antigens in the CML assay support the concept that such quantitative differences may lead to a differential antigenic function of LD and SD in the complex sequence of events present in the in vitro response to allogeneic cells. This is the important question biologically. The findings reported here are not inconsistent with this.

Summary

The cell-mediated lympholytic capability of mouse spleen cells stimulated in mixed lymphocyte culture is determined by lymphocyte-defined (LD) and serologically-defined (SD) antigenic differences present during sensitization. Cells which are activated by LD differences alone are markedly less effective in causing lysis of target cells. This lack of cytotoxicity is shown to be, at least in part, due to the inability of LD differences to allow the efficient generation of cytotoxic lymphocytes. SD antigens not only serve as good targets for CML but are also shown to be important for the generation of cytotoxic lymphocytes during the mixed lymphocyte culture.

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