HLA-D REGION-ASSOCIATED DETERMINANTS SERVE
AS TARGETS FOR HUMAN CELL-MEDIATED LYSIS*

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After in vitro mixed lymphocyte culture, cytotoxic lymphocytes develop which have specificity for components of the main histocompatibility complex (MHC)[1] of the stimulating lymphocyte population (1, 2). The effector cells were found to be directed mainly against class I histocompatibility antigens, that is, the H2-K and H2-D antigens in the mouse (3), and the HLA-A or HLA-B antigens in man (4). Cytotoxicity against HLA-C was quite weak (5). The role of class II antigens was thought to be that of providing a necessary amplifying effect (6, 7), however, it became clear later that incompatibility at HLA-D was not necessary for cell-mediated lysis (CML) to occur (8). In mice, class I antigens (H2-K and H2-D) alone, or class II antigens (H2-I) alone, were able to generate strong CML (9).

Most investigators who have performed studies with human lymphocytes have concluded that HLA-D region products do not serve as targets for CML (6, 7, 10). However, cytotoxicity directed at MHC products other than the known class I (HLA-A, B, or C) specificities has frequently been observed (8, 11–13). In spite of the failure to detect cytotoxicity against HLA-D products, the question of their role was not considered resolved by several authors (14–16). In view of the analogy between HLA-D and the I region in the mouse, reports that products of H2-I serve as strong targets for CML (17–19) were particularly relevant to this question.

In the present experiments the possibility that products of the HLA-D region may act as target antigens in CML was investigated by using monocytes as target cells. The effector cells were generated in vitro by culture with stimulating cells from unrelated donors, matched in the HLA-A and B regions, but mismatched for HLA-D. The results obtained with a variety of target cells, showed that cytotoxicity correlated with HLA-D, as determined by serologic typing. Specific blocking experiments with unlabeled monocytes have confirmed that products of HLA-D were recognized by the cytotoxic lymphocytes.

Materials and Methods

Study Subjects.   Blood donors used in these studies were members of our permanent tissue typing panel. They have been typed for HLA-A, B, C, D, and DR, repeatedly over a period of years.

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[1] Abbreviations used in this paper: CML, cell-mediated lysis; HTC, homozygous typing cells; CML, cell-mediated lysis.

HLA Typing. The typing reagents used included local sera, sera obtained by exchange with other investigators, and those exchanged in the 7th International Workshop. Testing for the HLA-A, B, C antigens was performed by using a standard microcytotoxicity method (20). The HLA-DR typing was performed on both purified B cells and monocytes as described in detail elsewhere (21, 22). The HLA-D typing was obtained by mixed lymphocyte culture with homozygous typing cells (HTC) as previously described (23).

Cell Preparation. Venous blood was drawn into heparinized containers and diluted with an equal volume of RPMI-1640 medium before centrifugation on Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo) as described by Böyum (24). The resultant peripheral blood mononuclear cells, were washed twice with RPMI-1640, before suspension in serum-enriched RPMI-1640 medium. Pooled normal human serum obtained from 40 healthy male donors, was used at either 20% concentration (RPMI-20% serum) or 10% (RPMI-10% serum).

Monocytes. Peripheral blood mononuclear cells in RPMI-20% serum were placed in 100 × 20-mm plastic dishes (Kimble, Div. of Owens-Illinois, Toledo, Ohio) and incubated overnight at 37°C, in a humid atmosphere containing 5% CO_2 and 95% air. By repeatedly washing the dish with RPMI-1640 medium, the nonadherent cells were removed. The adherent cells were then resuspended in RPMI-20% serum with the aid of a rubber policeman. Such cells were greater than 95% viable by trypan blue exclusion and 70-80% ingested latex particles. Euchrysine staining (22) showed 70-80% of the cells to have the red cytoplasmic fluorescence and bean shaped nuclei characteristic of monocytes.

B-Cell Lymphoblastoid Cell Lines. Peripheral blood mononuclear cells from selected HLA-D homozygous donors, were transformed by using the B95-8 strain of the Epstein-Barr virus (25) with the kind help of Dr. Graham Smith, Department of Internal Medicine, University of Texas Health Science Center at Dallas. After 3-5 wk, the cell lines with characteristics of B lymphocytes were established in culture. Cells were stored frozen in liquid nitrogen and thawed for use as needed for these experiments.

Generation of Cytotoxic Effector Cells. Peripheral blood mononuclear cells from selected donors, were cultured with stimulating cells, which had been X-irradiated (3,000 rads from a ^{137}Cs source) in 12 × 75 mm round-bottomed tubes (BioQuest, BBL & Falcon Products, Cockeysville, Md.). 3 million responder cells and 1.5 × 10^6 stimulator cells were cultured in 3.0 ml RPMI-10% serum at 37°C, in a humid atmosphere of 5% CO_2, 95% air. Responder cells were also cultured in the presence of autologous X-irradiated cells for use as controls. After 6 d of culture the cells were resuspended and adjusted to a concentration of 10 × 10^6 per ml for use in the cytotoxicity assay.

Cytotoxicity Assay. Monocytes to be used as target cells were labeled with radioactive sodium chromate ($Na_2^{51}CrO4$, Amersham Corp., Arlington Heights, Ill.). To 5-10 × 10^6 monocytes in 0.2 ml RPMI-20% serum was added 200 μCi of ^{51}Cr. The cells were then incubated at 37°C, for 1 h with occasional shaking, washed three times with RPMI-20% serum at 4°C, and suspended at a concentration of 10^5 cells per ml in RPMI-10% serum.

The cytotoxicity assays were performed in round bottom microtiter plates (Linbro Chemical Co., New Haven, Conn.). Each reaction well contained 5 × 10^3 target cells and 5 × 10^5 effector cells, or autologous controls, in a total vol of 0.2 ml. After incubation at 37°C, for 6 h, the radioactive supernates were collected into absorbing elements of cellulose acetate (Titertek, Flow Laboratories, Inc., Rockville, Md.) and counted in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Inhibition by Unlabeled Cells. In some experiments, cells that were not labeled with radioactive chromium were added to the cytotoxicity assay, to determine their effect on target cell lysis. The unlabeled cells were added to the microtiter wells at the beginning of the cytotoxicity assay. Their number varied in different experiments from 5 × 10^4 to 4 × 10^5 per well.

Calculation of Results. The results of CML experiments were expressed as percent cytotoxicity, according to the formula:

$$\text{percent cytotoxicity} = ABx.T^* - AAx.T^*/Max.T^* - AAx.T^* \times 100,$$

where ABx.T* represents ^{51}Cr released from target cell T* by the effector cells stimulated by allogeneic cells, AAx.T* represents the spontaneous release due to cells from the same responder cultured with autologous stimulators and Max.T* is the maximal release of radioisotope from target cells obtained by addition of 0.1 ml of undiluted 7× detergent. The mean maximal

release in these experiments was 89 ± 1.3% of the total found radioactivity. Spontaneous release in these experiments equalled 23 ± 0.9%.

In some experiments in which the inhibition of CML was investigated, results were also expressed as percent inhibition. This was calculated as follows:

percent inhibition = (1 − [percent cytotoxicity with unlabeled cells/

percent cytotoxicity without unlabeled cells]) × 100.

When cytotoxicity was not inhibited or it was increased after addition of the unlabeled cells, the percent inhibition was expressed as zero.

Statistical evaluation of the data was performed by chi-square analysis with Yates' correction and by the Student's t test.

Results

Generation of Effector Cells by Culture with Stimulating Lymphocytes Mismatched for the HLA-D Region. To determine whether effector cells can be generated in vitro by culture with cells compatible except for HLA-D, responding cells R.M. were stimulated in vitro by cells from an unrelated donor G.S. The HLA phenotype of R.M. was HLA-A1, A2, B8, B18, DW2, DW8, DRW2, WIa8, and G.S. was known to be homozygous HLA-A1, B8, DW3, DRW3 on the basis of family and mixed lymphocyte culture studies. No HLA-C alleles were detectable in the stimulator or the responder. To determine whether cytotoxic lymphocytes developed against determinants of the HLA-D region, a panel of DRW3-positive and DRW3-negative individuals was selected for testing. The details of HLA-A, B, C, and DR antigens detected in these persons by serologic typing is shown in Table I. The results of cell-mediated lysis experiments with chromium-51-labeled target monocytes are shown in Table II. Cytotoxicity correlated with the presence of DRW3. When DRW3 was absent from the target cells, cytotoxicity was between 5 and 13%. In contrast, the same effector cells produced much higher chromium release (53–76%) from the monocytes of the DRW3-positive donors.

Cell-Mediated Lysis with Other Effector Cells Directed against the HLA-D Region. In Fig. 1, the first effector cell was developed by culturing cells from responder R.M. and the DW3, DRW3 homozygous stimulator, J.G. The results show that cytotoxicity was present when the target cells were DRW3 positive like the stimulator.

The effector cells in the second experiment shown in Fig. 1, were generated between responder R.G. (HLA-A1, A2, B8, B12, DRW1, DRW3) with stimulating cells J.W. (HLA-A1, A2, B8, B12, DRW4). The stimulating antigen in this combination was DRW4 for which J.W. was homozygous. The test panel consisted of four donors having DRW4, who gave cytotoxicity between 27 and 50% and three donors who were DRW4 negative, whose cells gave very low levels of chromium release (4–11%). In this series of experiments 20 target cells were DRW3 positive and the mean cytotoxicity was 46.5 ± 2.9%. The mean cytotoxicity of 14 target cells negative for DRW3 was only 6.4 ± 1.1%. The difference between the means of the two groups was highly significant ($P < 10^{-4}$). Similarly, with effector cells primed against DRW4 there was a highly significant difference ($P < 0.001$) in cytotoxicity between target cells having the HLA-D determinant matched to the stimulator (30.0 ± 4.7%) compared to those who did not (3.8 ± 1.8%).

Role of HLA-D and D.R. in Cell-Mediated Lysis. It is possible to identify specificities coded by the HLA-D region by serologic and by mixed lymphocyte culture typing. Serologic typing of isolated B lymphocytes allows identification of broad HLA-D

TABLE I

HLA Phenotypes of Donors of Target Cells used in Table II

Target cells	HLA Antigens			
	A	B	C	DR
P.D.	W24, —	W35, W37	4, 6	W3, W4
L.K.	1, 2	7, 8	6, —	W3, —
P.P.	1, W24	8, —	—, —	W3, —
B.B.	1, —	9, —	—, —	W1, W3
C.K.	1, 3	7, 8	—, —	W1, W3
L.Mc.	W32, —	14, W40	—, —	W7, —
P.S.	3, 11	14, W38	—, —	W1, —
H.S.	1, —	12, 17	5, 6	W6, W8
S.G.	1, 2	5, 12	—, —	W2, W4
R.M.	1, 2	8,18	—, —	W2, W8

TABLE II

Cell-Mediated Lysis of Target Monocytes by Effector Cells Exposed to HLA-DRW3 and Associated Determinants of the HLA-D Region *

Target cells	DR W3‡	Radioactivity released§			Cytotoxicity‖
		Maximal	Spontaneous	Experimental	
		cpm			%
P.D.	+	2,655	491	1,639	53
L.K.	+	2,232	381	1,510	60
P.P.	+	1,907	478	1,575	76
B.B.	+	3,236	554	2,281	64
C.K.	+	2,340	386	1,671	65
L.Mc.	—	2,171	393	524	07
P.S.	—	2,474	428	597	08
H.S.	—	2,703	545	821	13
S.G.	—	2,473	445	549	05
R.M.	—	2,228	378	515	07

* Effector cells were generated by culturing responder R.M.(HLA-A1, A2, B8, B18, DRW2, WIa8) with stimulating cells G.S.(HLA-A1, A1, B8, B8, DRW3, DRW3).

‡ Complete HLA typing given in Table I.

§ Target cells were labeled with Na_2 $^{51}CrO_4$; maximal release was obtained with detergent; spontaneous release determined in mixtures of labeled target cells with RM·RM$_x$ control effectors, spontaneous and experimental assay mixtures were incubated for 6 h.

‖ Percent cytotoxicity calculated by using the formula given in text.

related (DR) antigens. The lymphocyte stimulating determinants identified by MLC typing with HTCs are more restricted. Thus some of our panel donors presented a given broad HLA-DR specificity without the HLA-D antigen (by MLC) which is usually associated with it in Caucasians. When cells from such D/DR discrepant donors were used as targets with effector cells stimulated against the HLA-D region, the results showed, that presence of the HLA-DR determinant was sufficient for cytotoxicity to occur. This was true also with target cell P.C., known by family study to have HLA-DW11, DRW4 on the paternal haplotype. Yet P.C. cells were killed by effector lymphocytes primed against a DW4, DRW4 stimulator. The magnitude of
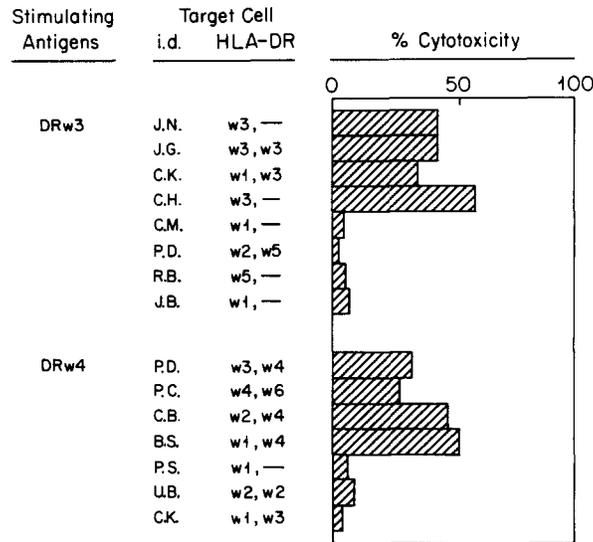
FIG. 1. Cell-mediated lysis directed against HLA-D region determinants. Effector cells were RM·JG$_x$ for DRW3 and RG·JW$_x$ for DRW4. Cytotoxicity appeared to correlate with presence of the stimulating DR determinant in the panel of unrelated target cells.
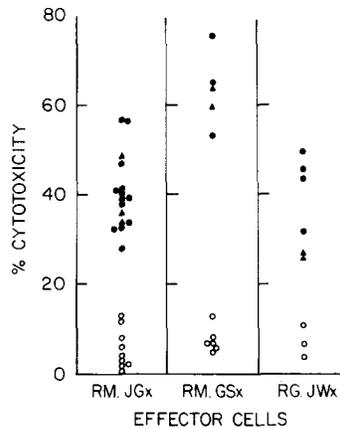
FIG. 2. Cell-mediated cytotoxicity against HLA-D region determinants. Effector cells RM·JG$_x$ and RM·GS$_x$ stimulated against DRW3, RG·JW$_x$ against DRW4. The target monocytes used in these experiments were from donors matched for the stimulating HLA-D and DR antigens ●, matched for the stimulating HLA-DR but not HLA-D (as determined by MLC typing) ▲; or not matched for either HLA-D or DR ○

cytotoxicity obtained when HLA-DR-positive, HLA-D-negative cells were used was similar to that obtained with target cells having both determinants matched with the stimulating cells (Fig. 2). The specificity of the cytotoxic effector lymphocytes thus appeared to correlate with target specificities recognized by serum antibodies, rather than with specificities determined in MLC by proliferating T cells.

Antigen Specificity of Inhibition by Unlabeled Monocytes and Cultured B Cells. Results of five separate experiments in which unlabeled monocytes were used to analyze the

TABLE III

Specificity of Blocking of Cell-Mediated Lysis against HLA-D Region-Associated Determinants by Unlabeled Monocytes

Exp.	Priming of effector cells*	Target cell DR	Blocking cell DR	Cytotoxicity	Inhibition
				%	%
1	DRW3	W3	—	33	—
			W2, W3	13	61
			W3, —	12	64
			W2, W4	41	0
			W1, W2	37	0
2	DRW3	W3	—	69	—
			W3, —	12	83
			W3, W3	7	90
			W2, W4	64	7
3	DRW2	W2	—	11	
			W2, —	0	100
			W4, —	10	9
4	DRW2	W2	—	13	—
			W2, W4	0	100
			W1, W3	14	0
5	DRW4	W4	—	11	—
			W2, W4	1	91
			W1, —	10	9

* Effectors in exp. 1 and 2 were produced as in Fig. 1; effector cells in exp. 3 and 4 were produced by priming a responder W.S. (HLA-A3, A11, B12, B15, C1, -DRW4, DRW7) with cells from stimulator U.B.(HLA-A3, A11, B7, -DRW2, DRW2), target cells and blocking cells in these experiments did not have HLA-B7; effector cells in exp. 5, were produced as in Table II.

specificity of the blocking effect are shown in Table III. In each case monocytes from unrelated donors, known to be positive for the HLA-DR antigens to which the cytotoxic cells were primed, were found to inhibit cytotoxicity. Monocytes from donors who did not have a HLA-DR antigen matched with the target cell were found not to block. The specificity of monocyte blocking was examined in experiments with unlabeled monocytes from 41 donors, by using effector cells primed against HLA-DRW2, DRW3, DRW4. Blocking was achieved 23 times when the antigen was present and no inhibition was observed in 14 tests, when the antigen was absent. One cell preparation having the correct antigen failed to inhibit. In three instances there was a false positive result. Two of the three cells which blocked despite absence of the sensitizing antigen (DRW4), belonged to an individual positive for DRW7. This antigen is known to cross-react with DRW4, the target antigen in this particular experiment. The chi square obtained from these results is 23 ($P < 10^5$) indicating that there was a strong correlation between the presence of a specific HLA-DR antigen and the capacity to inhibit CML.

Similar results were obtained when cultured lymphoblastoid cells were used instead of isolated monocytes. These cell lines are known to have the characteristics of B cells, including the presence of HLA-D and HLA-DR specificities. Only the cell lines having the correct HLA-DR specificity were found to be capable of inhibiting the chromium release. The correlation between CML inhibition and the HLA-DR antigens in monocytes and B cells from unrelated donors strongly suggests that the HLA-D region product itself was responsible for the inhibitory effect.

Discussion

Previous investigators have either failed to find cytotoxicity associated with HLA-D, or were unable to draw firm conclusions regarding this point (14–16). In the present experiments, effector cells were generated among HLA-A and B region compatible, HLA-D region incompatible, unrelated individuals. Because of identity in the HLA-A and B regions, effector cells obtained from such cultures could be directed against determinants encoded by the HLA-D region, or against other determinants which are not identified by current typing methods. The stimulating cells were HLA-D homozygous so the effectors were primed only against one HLA-D specificity. In some of the cells used, no HLA-C alleles were detectable with antisera identifying HLA-CW1 through CW6, but it seems unlikely that HLA-C associated determinants would have played an important role. Because HLA-C and HLA-B alleles are in strong linkage disequilibrium, there is a good chance, if the HLA-B alleles were matched, that the C alleles would have been matched also. In addition, products of HLA-C are known to be poorly antigenic (26) and known not to be strong targets for CML (5).

The evidence from the present experiments that HLA-D associated products serve as targets for cell mediated cytotoxicity are in contrast with multiple previous reports to the contrary by other investigators (6, 7, 10). It should be remembered however, that beginning with the report by Lightbody et al. (2) most human CML experiments have been carried out by using human T cells undergoing blastic transformation after culture with phytohemagglutinin. Although there is general agreement that the majority of T lymphocytes obtained from human peripheral blood do not express the HLA-DR determinants there is controversy as to whether or not these antigens appear in human T cells during blastic transformation after in vitro culture with mitogens. Colombani et al. (27) have reported that human T-cell blasts obtained after culture with phytohemagglutinin or concanavalin A, express HLA-DR specificities detectable by complement fixation. Only a small population of concanavalin A-induced blasts in the mouse was reported to be killed by anti-Ia serum and complement (28). In the present experiments the target cells were peripheral blood monocytes isolated by adherence to plastic dishes. It is well established that human monocytes, obtained in this manner, express HLA-DR antigens (29, 30) and it is known that monocytes are sensitive targets for chromium release cytotoxicity assays (31).

Our results showed that using a panel of unrelated target cells the cytotoxicity reactions correlated with the HLA-DR antigens of the target cell donors. Effector cells used were primed with cells mismatched for HLA-DRW2, DRW3, or DRW4. To be killed, the donors of the target cells had to possess the HLA-DR antigen to which the effector cell had been exposed in the sensitizing culture. These results were interpreted to mean that cytotoxicity was directed against HLA-D region products or very closely linked determinants.

The cytotoxicity inhibition experiments with unlabeled target cells, lend further support to the notion that HLA-D region associated determinants were involved in these cytotoxicity reactions. Blocking with monocytes from unrelated donors was found to depend on the presence of the correct HLA-D alleles. The correlation of HLA-DR typing of the monocytes capable of blocking, with that of the target cells, was observed for DRW2, DRW3, and DRW4. One interesting exception was a false positive result obtained in an experiment in which cells from a donor positive for HLA-DRW7 were found to block cytotoxicity presumed to be directed against HLA-

DRW4. DRW4 and DRW7 antigens are known to be cross-reactive when examined by using antibodies against B lymphocytes (32). The observation of similar cross-reactivity in cold-cell blocking experiments suggests that some of the cytotoxic effector cells were recognizing determinants shared by DRW7 and DRW4.

At the present time HLA-D region alleles are recognized by mixed lymphocyte culture typing (33), by primed lymphocyte culture typing (34), and by serologic typing with isolated B cells or monocytes (22, 32). It is not known whether one or several gene products are involved. From the results it is not possible to decide whether the HLA-D region determinants recognized by cytotoxic lymphocytes are the same as those recognized by the other techniques. The HLA-DR specificities are known to be broader than the HLA-D determinants defined by typing with homozygous typing cells (35). Thus a number of donors used as CML targets were HLA-DR positive HLA-D negative. Monocytes from such donors were found to be good targets as long as the correct HLA-DR specificity was present.

The results of the present studies, together with the finding that antibodies against HLA-DR effectively inhibited the cytotoxicity reactions,[2] suggest but do not prove, that the same HLA-DR products were recognized by antibodies in the typing sera and by the cytotoxic lymphocytes. Cell mediated lympholysis has been considered to be an in vitro model of allograft rejection (9). The inhibition of cytotoxicity by anti-DR antibodies as observed in the present experiments, may be a model of what happens in vivo during antibody enhancement of experimental grafts (36). Perhaps the HLA-DR specificities serve as targets for cytotoxic lymphocytes in vivo as well as in vitro.

Summary

Effector cells for cell-mediated lysis (CML) were generated by in vitro culture of lymphocytes from selected donors with X-irradiated cells from unrelated subjects who were HLA-D homozygous and matched to the responders for the antigens of the HLA-A and HLA-B regions. By using chromium labeled monocytes as target cells, cytotoxicity was found to correlate with presence of HLA-D region antigens matching those of the stimulating cells. Such CML reactions apparently directed at products of HLA-D, were inhibited by addition of unlabeled monocytes or B lymphocytes. These unlabeled cells had to be matched for HLA-D with the stimulating cells used to generate the effector populations. The results suggested that products of HLA-D, perhaps the DR antigens, were recognized by cytotoxic lymphocytes.

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