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HLA Restriction of non-HLA–A, –B, –C and –D Cell Mediated Lympholysis (CML)

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The aim of our study was to define target determinants other than those coded for by the classical HLA–A, –B, –C or –D loci which were responsible for killing in CML. In one of the families studied, strong evidence was found for the existence of a determinant coded for within the HLA region. CML was restricted to targets carrying the classical HLA–Bw35 and Cw4 determinants but the targets were neither HLA–Bw35 nor Cw4 themselves. We therefore concluded that this new HLA determinant was either the product of a new locus closely associated with HLA–B or that it was a product of the classical HLA–B locus which has not been recognized by serology.

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It is generally accepted that a sensitization step is required for the generation of effector cells in cell mediated lympholysis (CML) (Lightbody et al. 1971, Eysvoogel et al. 1973a, b, Trinchieri et al. 1973). However CML can be obtained after *in vitro* sensitization between HLA–D identical individuals (Goulmy et al. 1975) and also after *in vivo* sensitization through allografting between individuals who give low or negative mixed lymphocyte reactions (MLR) (Mawas et al. 1973b, Parkman et al. 1976).

Differences appear to exist in the specificity of CML according to whether the sensitization step was conducted *in vivo* or *in vitro*. After *in vivo* sensitization through skin or bone marrow transplantation target determinants associated with both HLA (Mawas et al. 1973a) and non-HLA can be shown (Mawas et al. 1973b, Parkman et al. 1976). After *in vitro* sensitization only target determinants associated with the HLA region have been reported. Depending on phenotypes of effector and sensitizing cells used *in vitro* the specificity of CML can be directed either to classical HLA–A, –B or –C targets (Lightbody et al. 1971, Eysvoogel et al. 1973a, b, Trinchieri et al. 1973, Kristensen et al. 1975b, Grunnet et al. 1976) or to other imprecisely defined targets (Bach et al. 1973, Kristensen et al. 1974, Mawas et al. 1974, Schapira & Jeannet 1974, Grunnet &

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Kristensen 1975, Grunnet et al 1975, Kristensen & Grunnet 1975, Kristensen et al 1975a, Mawas et al 1975, Takasugi et al 1975)

In other species there is increasing evidence that genetically coded membrane structures and membrane associated viral and synthetic structures are restricted in their ability to behave as targets in CML by a requirement for the target cell to carry the H-2 specificity of the sensitizing cell (Blanden 1974, Shearer 1974, Zinkernagel & Doherty 1974, Bevan 1975, Gartner et al 1975, Gordon et al 1975, Schrader et al 1976) Thus it remains to be established which locus codes for those target determinants which are independent of the classical HLA-A, -B, or -C antigens and are yet associated with the HLA region Are they non-HLA targets whose performance is governed by HLA coded products or are they simply new, as yet unrecognized targets coded for within the HLA region? In this work we present a family study in which CML is directed towards determinants other than the classical antigens HLA-A, -B, -C and -D The segregation patterns of the CML reactions support the view that the targets involved are coded for by a locus within the HLA region

Materials and Methods

Lymphocyte donors Healthy HLA-typed donors were used All individuals were typed at least twice for HLA-A, -B and -C and in some cases for HLA-D locus products using the positive typing methods (PLT) (Sheehy et al 1975, Bradley et al 1976, Termijtelen & Bradley 1976) For 10 family studies, discrete haplotypes were identified as shown in the example in Table 1

Lymphocyte preparation Peripheral lymphocytes were obtained from heparinized blood, separated through a ficoll-isopaque gradient The MLC tests were set up in a micro technique (Hartzmann et al 1971)

We used a modified CML technique described by Alter et al (Personal communication)

Table 1

Family van R Haplotypes

	Paternal a	Paternal b	Maternal c	Maternal d	Positive typing (PLT) for HLA-D ψ	DW1 ◊
Father	A2,BW35,W6 Φ,CW4	*A2,BW35,W6,CW4*			1	N T
Mother			A2,B12, W4	A11,B5, W4	2	N T
C1,C2	A2,BW35,W6,CW4		A2,B12, W4		3	2
C3,C4		*A2,BW35,W6,CW4*	A2,B12, W4		1	2
C5,C6		*A2,BW35,W6,CW4*		A11,B5, W4	1	2
C7	A2,BW35,W6,CW4			A11,B5, W4	N T	N T

Φ W6 was formerly 4b

ψ C2 was sensitized to C4 and tested in a secondary MLR Score 1 indicates that the presumed HLA-I determinant of C4 was shared by that cell, scores 2 and 3 indicate absence of that HLA-I determinant (see ref Termijtelen & Bradley 1976)

◊ See key Table 6

Primary cultures: Sensitization of effector cells: 8×10^6 responder cells and 16×10^6 stimulator cells (irradiated 2,000 R) were cultured in a total volume of 20 ml culture medium (RPMI-1640, 20 mM L-glutamine, 100 I.U. penicillin/ml, 100 μg streptomycin/ml) and incubated in a humidified CO_2 2,5% incubator (Hereus type KB 500 CO_2) in a 45° position in tissue culture flasks (Falcon no. 3012) for 6 days. Then effector cells were collected from the bottles, centrifuged 10′ at 350 g and resuspended in 2 ml culture medium. Viability counting was performed in eosin. Effector cells were finally suspended in a concentration of 7×10^6 viable cells/ml.

Target cells: 8×10^6 cells (in duplicate) were cultured in 8 ml of culture medium in tissue culture flasks. After 3 days 50 λ PHA Difco-M stock solution was added to each bottle of target cells. On day 6 the target cells were collected from the bottles and centrifuged at 150 g for 10′. Target cells were resuspended in 1 ml of their own supernatant and incubated for 1 h at 37°C with 250 μCi ^{51}Cr (^{51}Cr Na_2CrO_4 5 mCi/5 ml; spec.act. 100-200 mCi/mg Amersham CJS IP). The targets were shaken several times during the incubation. After incubation ± 4 ml washing fluid (Hanks' BSS supplemented with penicillin and streptomycin) was added, gently mixed and centrifuged 10′ at 150 g, the cells were gently washed twice, and resuspended in 5 ml culture medium. Viability counting (with eosin) was done and a suspension of 1×10^5 cells/ml was made.

Cell Mediated Lympholysis (CML) assay: The effector cells and the labeled target cells were incubated together in roundbottomed microtiter plates (Cooke M220-24 AR). 0.1 ml of each suspension was used, resulting in an effector:target ratio of 70:1. For measuring the spontaneous release 0.1 ml of culture medium was added to 0.1 ml of each target cell suspension. All combinations were tested in triplicate. The plate(s) were spun for 2′ at 200 g, and incubated for 4 h at 37°. After incubation 0.05 ml culture medium was added to each well. 0.25 ml of each freeze-thaw target (see below) was used in triplicate. The plates were spun for 5′ at 500 g. The supernatants were harvested without any cells and counted for 5′ per sample, in a gammacounter (Packard autogamma scintillation spectrometer 5260).

Freeze-thawing for maximum release: 1 ml of target cells and 1.5 ml H_2O were put into a culture tube. The freezing was done in liquid nitrogen, the thawing in a waterbath of 37°C. This was repeated 3 times, then the cells were spun for 10′ at 500 g and the supernatant was used.

Analysis and interpretation of results: Raw counts were used. The results are expressed in % of specific ^{51}Cr release, according to the formula:

$$\frac{\text{experimental mean} - \text{mean of spontaneous release}}{\text{freeze thaw mean} - \text{mean of spontaneous release}} \times 100\% = \%\ \text{kill}$$

The variation coefficient within the triplicates was always less than 10%.

Results

A search was made for CML target determinants coded for by genes outside the HLA complex and to this end we tested 10 families. Cells of the progeny were sensitized against each parent and 40 such cells were tested against MHC identical siblings. We found no cytolysis in nine of these families which could be attributed to non-classical HLA–A, -B, or -C antigens but in one family (van R.) we found strong killing between several identical siblings.

Family van R.

HLA haplotyping for this family is shown in

Table 2
Stimulation index in MLR of family van R

	C1	C2	C3	C4	C5	C6	C7	F
$C1_x$	1 0	1 0	8 2	4 6	14 4	13 3	15 4	13 7
$C2_x$	1 3	1 0	12 1	6 8	17 5	19 1	18 8	18 5
$C3_x$	16 5	15 6	1 0	1 4	13 3	12 6	9 4	13 1
$C4_x$	10 2	10 3	2 0	1 0	6 0	8 0	11 0	12 3
$C5_x$	22 1	17 9	8 6	6 6	1 0	0 6	7 2	11 7
$C6_x$	27 8	28 9	15 7	12 0	1 4	1 0	6 2	20 4
$C7_x$	21 1	22 4	17 1	12 0	8 1	8 9	1 0	17 9
$Father_x$	31 1	34 9	15 1	12 0	19 8	17 3	28 0	1 0

Table 3
CML results of family van R showing 7 siblings against the cells of the father

		SI in MLR against father	7 kill on father 2 W35 CW4/ 5 / 0 m4
C1	a	31 1	40
C2	ac	34 9	44
C3	bc	15 1	25
C4	bc	12 0	18
C5	bd	19 3	16
C6	bd	17 3	30
C7	ad	28 0	58
F	ab	1 0	1

No autologous kill occurred

Table 1 The father was homozygous for HLA–A, –B and –C but heterozygous for HLA–D since this was indicated by the PLT typing result shown to the right side of Table 1 This was further confirmed by the segregation studies of the mixed lymphocyte reaction (MLR) as shown in Table 2 Thus four haplotypes – a, b, c and d were defined

In Table 3 the MLR and CML results from seven siblings sensitized against the cells from the father are shown All siblings recognized some target determinants on the father's cells, which led to cytolysis However no detectable HLA differences defined by serology existed A number of cell combinations in this family was tested, and these are shown in Table 4 In the combinations where the stimulator differed from the responder by the paternal a-haplotype, CML was directed only to targets carrying the a-haplotype Similarly where the stimulator differed from the responder by the paternal b-haplotype, CML was directed only to targets carrying the b-haplotype No paradoxical CML patterns were obtained in the nine combinations tested Thus it seemed extremely unlikely that the target determinants were coded for by non-MHC loci In the ninth combination (C2 $C5_x$) killing occurred both against HLA–A, –B and –C identical but MLR different siblings C3 and C4, and against C5 and C6 The higher killing against the latter was attributed to additional sensitization to the foreign maternal haplotype

The next step was to test the sensitized cells on an unrelated panel (Table 5 expt 1) Child 1 sensitized to child 3 tested on 12 individuals was able to lyse six out of seven Bw35 Cw4 positive cells and no Bw35 Cw4 negative cells Cells from child 3 sensitized to child 1 did not recognize any determinants in this panel which led to cytolysis In the next experiment, child 6 with child 7 was tested on 16 unrelated cells The results are shown in Table 5, expt 2 Combination C6 $C7_x$ showed lysis on two out of 13 Bw35 Cw4 positive cells and combination C7 $C6_x$ was able to lyse the remaining 11 out of the 13 Bw35 Cw4 positive cells Thus Bw35 Cw4 positive target cells which were not lysed by one combination, were lysed by the reciprocal combination (C6 $C7_x$)

Therefore it appeared that positive cytolysis was through the recognition of an HLA–Bw35 or Cw4 associated target determinant Because of the high linkage between Bw35 and Cw4, Bw35 could not be tested independently of Cw4 In Table 6 the HLA phenotypes of the targets are listed HLA–A2 was not relevant as a target for the lysis because many HLA–A2 negative cells were killed In addition Cw4 did not appear to be relevant as a target since four out of 37 Cw4 positive targets were CML negative

Table 4

CML results of 2 experiments in family van R [ψ]

Responder x stimulator (sensitizing haplotype)	S.I. in MLC	target cells haplotypes C1 ac	C2 ac	C3 bc	C4 bc	C5 bd	C6 bd	C7 ad
C4 x C1 (a)	4.6	14	13	2	5	3	[φ]N.T.	11
C3 x F (a)	15.1	16	N.T.	3	6	N.T.	N.T.	16
C6 x F (a)	17 3	23	N.T.	7	11	N.T.	9	26
C1 x C4 (b)	10.2	3	0	33	37	N.T.	N.T.	2
	8.5	-1	3	11	7	19	17	1
C1 x C3 (b)	16.5	4	0	33	39	34	N.T.	N T.
C1 x F (b)	31.1	0	-1	N.T.	N.T.	N T.	N.T.	N T.
C2 x F (b)	34.9	1	0	N.T.	46	N.T.	N.T	4
	19.0	-1	-2	13	10	17	15	1
C7 x F (b)	20.8	7	N.T.	N.T.	59	N.T.	N.T.	1
	16.6	-2	N.T.	N.T.	13	22	15	-1
C2 x C5 (b,d)	17.9	1	-1	36	39	53	64	43

[ψ] Figures represent % Cr-release
If a combination was tested twice, the results from the second experiment are shown in the lower line The overall percentages in the second experiment were lower than in the first, but consistent with the killing pattern in the first experiment

[φ] N T = not tested

Table 6
HLA phenotypes of the unrelated panel (see table 5)
(case 1 and 2 of table 5)

		HLA-A		HLA-B		HLA-C	[x] Positive typing (PLT) for HLA-DW1
Expt. 1	Target 1	1	3	8	W35	CW4	1
	" 2	1	3		W35	CW3 CW4	1
	" 3	1	2	18	W35	CW4	1
	" 4		9	7	W15	CW3	2
	" 5	1	28	W40	27	N.T.	N.T.
	" 6		3	18	W17	-	N.T.
	" 7	1	9	8	W40	CW3	N.T.
	" 8	2	3	7	W35	CW4	2
	" 9	1	3	W35	W40	CW2 CW4	1
	" 10		3	14	W35	N.T.	N.T.
	" 11	1	3	7	8	-	N.T.
	" 12	1	9	18	W35	CW4	1
Expt. 2	Target 1	28	29	7	W15	CW3 CW4	N.T.
	" 2	10	28	(weak)W35	HR	CW4	2
	" 3	2	11	HR	W15	CW4	N.T.
	" 4	1	3	8	W35	CW4	N.T.
	" 5	2	3	W35	W22	(weak)CW4	1
	" 6	W24	W33	W35	14	CW4	N.T.
	" 7	1	11	W17	W15	CW3	N.T.
	" 8	2	11	W35		CW4	2
	" 9	2		W35	W16	CW4	N.T.
	" 10	2	3	W35	W40	CW3 CW4	N.T.
	" 11	2		W35	13	CW4	N.T.
	" 12	3	W31	W35	W40	CW3 CW4	N.T.
	" 13	2	3	W35	W17	CW4	1
	" 14	3	10 W26	W35		CW4	1
	" 15	3	W32	W35		CW4	1
	" 16	1	3	HR	W35	CW4	2

[x] Cells were tested against 3 different PLT cells which were specifically sensitized to HLA-DW1. Score 1 indicates DW1 is present, scores 2 and 3 indicate the absence of DW1 (see ref. Termijtelen & Bradley 1976).

Table 5

CML results against unrelated BW35 positive and negative targets.

Expt. 1.

Targets *	1	2	3	4	5	6	7	8	9	10	11	12	C1	C3	
BW35	+	+	+	-	-	-	-	+	+	+	-	+			
C1 $C3_x$ (b)	43	42	46	4	8	9	4	43	43	43	0	ψ 8	-1	46	% kill
C3 $C1_x$ (a)	-2	-2	0	-1	-1	-1	-1	-2	-1	-2	-2	7	15	-4	

Expt. 2.

Targets	1	2	3^{Φ}	4	5^{x}	6	7	8	9	10	11	12	13	14	
BW35	-	$(+)^{o}$	-	+	+	+	-	+	+	+	+	+	+	+	
C6 $C7_x$ (a)	4	3	2	2	0	19	1	2	2	1	2	1	1	-2	% kill
C7 $C6_x$ (b)	7	27	24	57	41	1	5	34	31	41	40	43	37	39	

Targets	15	16	C6	C7	
BW35	+	+			
C6 $C7_x$ (a)	15	1	-1	14	% kill
C7 $C6_x$ (b)	2	45	40	-3	

Φ Although BW35 negative this individual carried the crossreactive antigen HLA-B HR. (Engelfriet et al. 1973).

o case 2. target 2 showed a weak BW35, but was positive for HR.

* HLA phenotypes of the targets is listed in Table 6.

ψ Consistant with high background with Cl $C3_x$ combination.

x Only weak CW4 detectable.

HLA-D as a target was excluded by the results obtained from positive typing (PLT) for HLA-D in which three typing cells which had been specifically sensitized to HLA-Dw1 were used The results of these tests are shown in Table 6 Although HLA-Bw35 is known to be in high linkage disequilibrium with HLA-Dw1 (Keuning et al 1975) a clear dissociation was found between our typing results for HLA-Dw1, HLA-Bw35 and positive CML Further more HLA-Dw1 was not found in this family (Table 1)

Discussion

These results indicate that the target determinant seen in our CML test was coded for by a locus in close association with the HLA-B and -C loci The possibility that this was a non-MHC coded target determinant which was restricted by a requirement for HLA identity between sensitizing and target cells at the HLA-B or -C loci was very unlikely since the CML reaction pattern segregated with the HLA haplotype of the target in the combinations tested By *in vitro* sensitization methods these non-MHC determinants have, until now, been undetectable We were, however, able to demonstrate killing independent of the known HLA-A, -B, -C and -D specificities directed against target determinants governed by genes mapping within the HLA region This was in accordance with the findings from others (Kristensen & Grunnet 1975, Kristensen et al 1976) These newly defined structures can function as strong target determinants

In family van R the father was homozygous for HLA-A, -B and -C but probably not for -D and for the presumed target deter minant The HLA-D specificites within this family have yet to be identified and, as described elsewhere, a positive MLR cannot be considered to completely rule out HLA-D identity (Keuning et al 1975, Brad ley & Termijtelen 1976) However famil members and most of the HLA-Bw35 Cw cells were LD typed by means of the PLT since Bw35 was shown to be in high linkag with Dw1 (Keuning et al 1975) The result however excluded HLA-D as being respons ible for the killing Thus, because lysis wa found between the HLA-A, -B and -C identical, MLR-positive siblings and th reaction pattern segregated with HLA in th unrelated panel, the target determinan appeared to be restricted by HLA-Bw3 and/or Cw4 (Table 4 and 5)

We suggest that 16 out of 19 of th HLA-Bw35 Cw4 positive individuals carrie a "common" target determinant, which wa identical to that carried on the paterna b-haplotype of the family In at least two o the exceptions, killing was obtained by th reciprocal combination in which sensitiza tion was directed to the paternal a haplotype Thus the target was restricted b the Bw35 Cw4 tandem of antigens, but wa apparently not Bw35 or Cw4 itself It migh be that the killing was dependent on th compatibility between effector, primar stimulator and target at the HLA-B or -C loci This could be explained either by th dual recognition hypothesis or by th interaction antigen hypothesis as discussed by others (Zinkernagel & Doherty 1974 Bevan 1975) By the first hypothesis, kille cells would have to recognize self (Bw35 and/or Cw4) before the killing via the actua target determinant could be initiated By th second hypothesis expression of the non HLA target determinant would be regulated by the Bw35 and/or Cw4 gene product The existence of such a determinant on the Bw35 Cw4 negative haplotype was not entirely excluded since only a small number of Bw35 Cw4 negative individuals were tested How ever, none of these showed positive CML

A second explanation would be that we were able to detect two different antigens

which were linked to Bw35 and Cw4 or actually coded for by the B locus itself and yet not detectable by contemporary serological methods.

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