Cytotoxic T Lymphocytes Directed Against HLA-Bw35-linked Target Determinants Show Differences in Sensitivity toward Antibiotics during Sensitization Period

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ABSTRACT *Recently we demonstrated that the outcome of human CML* was influenced by the presence of antibiotics in the culture medium In earlier studies we had found that cytotoxic T lymphocytes could recognize HLA-Bw35-linked target determinants These so called Bw35 a and b determinants showed Mendelian segregation*

We describe in this article a difference between the Bw35 a and b cytotoxic T cells in their sensitivity toward antibiotics The lysis against the Bw35 b cytotoxic determinant was not influenced by either the presence or the absence of antibiotics during the sensitization period whereas the lysis against the Bw35 a cytotoxic determinant was drastically diminished when the effector cells were cultured in the absence of antibiotics during the sensitization period

ABBREVIATIONS

CML	cell mediated lympholysis	MLC	mixed lymphocyte culture
CTL	cytotoxic T lymphocyte	PHA	phytohemagglutinin

INTRODUCTION

Antibiotics such as penicillin and streptomycin are widely used in tissue culture media to prevent bacterial infection This is also the case in human cell typing techniques such as MLC* and CML* Recently, we demonstrated that the outcome of a human CML test was influenced by the presence of antibiotics in the culture medium Allogenic CTLs* cultured in the presence of antibiotics during the induction phase showed significantly stronger lysis against the specific target cells in comparison to CTLs cultured in the absence of antibiotics Furthermore, in some cases the CML assay was positive (according to the European CML Workshop criteria) [1,2] only when antibiotics were added to the cultures, whereas no lysis could be detected in the absence of antibiotics [3]

In 1976, we described the reaction pattern of two CTLs that recognized a determinant linked to but different from HLA-Bw35 and Bw53 These effector cells were raised in vitro between A-, B-, and C-identical but D-nonidentical siblings [4]

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The demonstration that antibiotics influence the outcome of CML has prompted us to investigate the effect of antibiotics on the generation of CTLs directed against HLA-Bw35/53-linked target determinants

MATERIALS AND METHODS

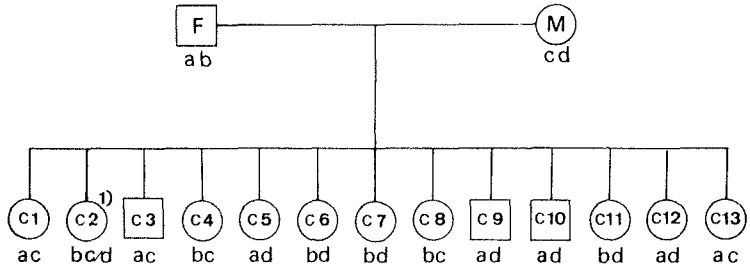
Lymphocyte donors Healthy HLA-typed panel donors were used Blood samples from family v R (2 parents and 13 children) were obtained at three different bleeding times The HLA genotypes of this family are shown in Figure 1

Lymphocyte preparation Peripheral blood lymphocytes were obtained from heparinized blood separated by Ficoll–Isopaque gradient centrifugation The separation and cryopreservation procedures were performed in antibiotic-free media

Generation of intrafamilial CTLs Responder cells (10^7) and 10^7 stimulator cells (irradiated with 2000R) were cultured in 25-cm^2 tissue culture flasks in RPMI 1640 medium containing 15% pooled AB serum supplemented with 25 mM HEPES and 3 mM L-glutamine When antibiotics were used, the standard amounts of penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to the cultures The responder and stimulator cells were cultured for 6 days at 37°C in a humidified CO_2 incubator Thereafter, effector cells were collected and the CML assay was carried out

Target cells Target cells were cultured for 6 days with or without antibiotics After 3 days 50 μl of PHA*-M stock solution (Difco) was added On day 6 the target cells were labeled with 100 μCi of ^{51}Cr for 1 hr at 37°C and washed three times before use

FIGURE 1 The HLA genotyping of family v R (*1*) HLA-A HLA-C crossing-over, the HLA type of this child is A2, Bw35, Bw51, Cw2, Cw4, DR2, LB5 (*2*) LB5 and LB5×8 are Leiden local assignments of DR5 splits



haplotypes

a	A2	Cw4	Bw35	LB5x8 [2)]
b	A2	Cw4	Bw35	LB 5 [2)]
c	A2	Cw5	Bw44	DRw8
d	A11	Cw2	Bw51	DR 2

CML assay CTLs and target cells were suspended in antibiotic-free culture medium Viability was assessed, and both CTL and target cell suspensions were resuspended at the desired concentrations The CTLs and target cells were incubated together in round-bottom microtiter plates for 4 hr at 37°C After incubation the supernatants were collected for gamma counting In most instances, triplicate wells were tested in at least four effector cell dilutions When cell numbers were limited, only one effector/target ratio (60 1 or 50 1) was used Spontaneous release was determined from the wells with target cells incubated in culture medium alone Maximum release was obtained from Zaponin (Coulter Electronics Ltd , Herts, England)–RPMI 1640 lysis of the target cells The percentage of lysis was calculated according to the following formula

$$\frac{\text{mean of experimental release} - \text{mean of spontaneous release}}{\text{mean of maximum release} - \text{mean of spontaneous release}} \times 100$$

Percentages equal to or below 10% lysis were considered negative when only one effector/target cell ratio was used Standard errors of the mean of triplicates were always less than 5%

RESULTS

Family Studies

The HLA genotyping of family v R is shown in Figure 1 Both paternal haplotypes carry the same serologically defined HLA-A, B, and C antigens but differ for the DR antigens The father is heterozygous for D as shown by MLC studies [4] Consequently, two paternal haplotypes can be identified, and CTLs can be induced between siblings sharing the same maternal haplotype (see Fig 1) Reciprocal lysis was obtained between the siblings that differed only in the paternal haplotype An example of reciprocal lysis and Mendelian segregation is shown in Table 1 Furthermore, the influence of the presence of antibiotics in the culture medium on the outcome of specific target cell lysis is presented as well Reciprocal CTL combinations were induced between siblings C3 and C8, cultured either with or without antibiotics during the sensitization phase As shown in Table 1, CTLs C3 C8x cultured in the presence of antibiotics showed lysis (varying between 39% and 53%) against target cells that carried the b haplotype When CTLs C3 C8x were generated in the absence of antibiotics, the lysis was equally strong In contrast, the reciprocal combination C8 C3x, sensitized in the presence of antibiotics, showed lysis against target cells that carried the a haplotype varying from 27% to 33%, whereas the same CTLs sensitized in the absence of antibiotics gave only 12–20% lysis No positive CML was obtained by both CTLs against autologous target cells or target cells that did not carry the corresponding haplotypes

Results similar to those shown in Table 1 were obtained in the second experiment using different bleedings from the family members (data not shown) Although the CTLs showed a lower degree of lysis in the second experiment, it was clear that the presence of antibiotics did not affect the generation of CTLs C3 C8x, whereas in the case of CTLs C8 C3x all combinations showed a reduction in lytic activity in the absence of antibiotics

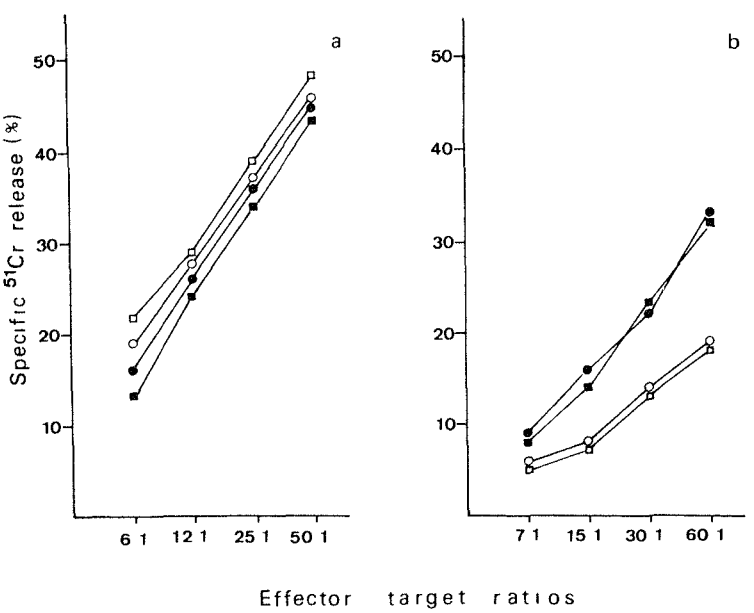
To determine whether specific lysis obtained with CTLs C8 C3x was significantly different when antibiotics were absent or present, additional experiments were set up and effector cell titrations carried out (Fig 2) CTLs were induced in either the presence or the absence of antibiotics and tested against target cells

TABLE 1 CML results in family v R

Target cell haplotypes	Specific ^{51}Cr release[a]			
	C3 C8x		C8 C3x	
	+[b]	−[c]	+	−
C3 (ac)	6	8	33	18
C8 (bc)	43	48	8	5
F (ab)	53	53	33	16
M (cd)	3	6	4	NT[d]
C1 (ac)	2	2	27	12
C2 (bc/d)	48	43	9	NT
C4 (bc)	44	56	10	NT
C5 (ad)	8	10	32	20
C6 (bd)	46	54	10	NT
C7 (bd)	39	53	9	NT
C9 (ad)	3	4	30	14

[a]The percent lysis of CTLs C3 C8x and C8 C3x are given for respectively 50 1 and 60 1 effector/target ratios
[b]CTLs were induced in the presence of antibiotics and tested against target cells cultured with antibiotics
[c]CTLs were induced in the absence of antibiotics and tested against target cells cultured without antibiotics
[d]Not tested

FIGURE 2. Reaction patterns of two intrafamilial CTLs against specific target cells in either the presence or the absence of antibiotics CTLs were induced in the presence of antibiotics and tested against specific target cells cultured with antibiotics (■) and without antibiotics (●) CTLs were induced in the absence of antibiotics and tested against specific target cells cultured with antibiotics (□) and without antibiotics (○) (a) CTLs C3 C8x, (b) CTLs C8 C3x



cultured with or without antibiotics In all titrations, the absence of antibiotics during generation of the CTLs C8 C3x (anti-a-haplotype) diminished the specific lysis (Fig 2b), whereas the absence of antibiotics had no influence on the generation of CTLs C3 C8x (anti-b haplotype) (Fig 2a) Furthermore, an influence of antibiotics on target cell cultures could not be demonstrated

Panel Study

Next the difference in susceptibility to antibiotics between the two HLA-Bw35 CTLs was tested against an unrelated panel selected for the presence or absence of the Bw35 antigen Reciprocal CTLs were raised between two other family members, namely, child 1 and child 4 As shown in Figure 1, child 1 and child 4 differ only in the paternal (A-, B-, and C-identical) haplotype Our earlier observation [4,5] of subdividing the serological defined Bw35 by CTLs led us to divide Bw35-positive panel members into two groups, called Bw35 a and b Table 2 shows the percentage of lysis by the CTLs against 7 family members and 19 unrelated panel members When the CTLs that specifically lyse the Bw35 a were cultured in the presence of antibiotics, target cells from family members (C1, F, C3, and C5) and unrelated panel members (1–7) were lyzed However, when no antibiotics were present in the sensitization phase, in each case lysis was diminished In contrast, no influence of antibiotics could be detected on the level of lysis when anti-Bw35 b-type CTLs were tested against Bw35 b-positive target cells (C4, F, C6, C8 and panel members 8–15) No lysis was obtained on the Bw35-negative target cells (panel members 16–19) in either the presence or the absence of antibiotics

DISCUSSION

In serology, the effects of chemotherapeutics and antibiotics on HLA typing have been described [6–11] Recently, a report from our laboratory demonstrated that, when lymphocytes were incubated with penicillin and washed before HLA typing, the cytotoxic reactivities of some typing sera with their corresponding antigen were blocked [12]

Furthermore, one of us showed that antibiotics such as penicillin and streptomycin could influence the specificity of CTLs in some responder–stimulator combinations [3] This is also the case in one type of HLA-Bw35-specific CTLs, as demonstrated in the present study

The two CTLs that showed specificity for HLA-Bw35-linked target determinants were induced in vitro between A-, B-, and C-identical siblings In this study we were able to demonstrate the difference in antibiotic susceptibility between the two different Bw35 CTLs Although we observed in different experiments variable degrees of specific lysis, in all experiments lysis by anti-Bw35 a CTLs was diminished when CTLs were induced in the absence of antibiotics The presence of a standard amount of penicillin and streptomycin during the 6-day sensitization phase seemed to be most critical This difference was not due to the infection of MLC cultures without antibiotics, since no bacteria were found in these cultures The presence of antibiotics in the target cell cultures did not influence specific lysis Once CTLs were induced, a negligible difference in specific lysis intensity was obtained whether target cells were cultured with or without antibiotics (Fig 2) We observed no influence of antibiotics on [^{3}H]thymidine incorporation in MLC cultures [3]

The mechanism by which the presence of antibiotics during the induction phase induces stronger cytotoxic effector cells than in the absence of antibiotics

TABLE 2 CML results against family and unrelated panel members

	Specific ^{51}Cr release[b]				
	C1 C4x		C4.C1x		
Target cells[a]	+	−[d]	+	−	Bw35 type[e]
Family members					
C1	−6	−2	22	6	a
C4	26	31	0	−6	b
F	40	43	36	21	a+b
C3	1	−2	32	13	a
C5	0	4	29	16	a
C6	30	41	−3	−3	b
C8	26	34	0	−3	b
Unrelated panel					
1	−1	0	23	13	a
2	7	8	24	15	a
3	2	1	23	13	a
4	5	4	12	7	a
5	3	2	15	8	a
6	1	1	18	11	a
7	7	7	29	17	a
8	45	50	5	4	b
9	35	33	0	0	b
10	37	42	2	0	b
11	46	43	4	0	b
12	32	39	0	0	b
13	30	35	1	1	b
14	30	34	0	1	b
15	30	32	2	1	b
16	0	2	3	4	−[f]
17	1	1	5	3	−
18	0	1	6	5	−
19	2	1	4	3	−

[a]All target cells were cultured in the presence of antibiotics

[b]The percentages lysis of CTLs C1 C4x and C4 C1x are given for, respectively, 50 1 and 60 1 effector/target ratios CTLs were induced in the presence of antibiotics

[d]CTLs were induced in the absence of antibiotics

[e]The HLA-Bw35 a and b types were established in earlier studies [4,5]

[f]HLA-Bw35-negative individuals

is at the moment still unclear. Several possibilities explaining these observations can be considered.

One possible explanation is that the combination of drugs and some HLA determinants during the induction phase of CTLs is more immunogenic than HLA determinants alone. A second possibility is that the drug renders the responder cells more efficient in recognizing some antigenic determinants during the induction phase of CTLs.

Whatever the mechanism is, it is clear that the presence of antibiotics in the induction phase can drastically alter the CML specificities. According to the European CML Workshop criteria, percentages of lysis equal to or below 10% are considered negative [1,2]. In the present study, we observed in all cases a

reduction in lysis in antibiotic-free cultures of HLA-Bw35 a-type CTLs, among these, some of the combinations even became negative (Table 2) This raises a valid argument for investigating the overall influence of antibiotics in cytotoxic assays Further studies are needed to investigate the drug effect on the sensitization phase of CTLs directed against HLA- and/or non-HLA

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