ALLOREACTIVE CYTOLYTIC T CELL CLONES WITH DUAL RECOGNITION OF HLA-B27 AND HLA-DR2 ANTIGENS

Selective Involvement of CD8 in Their Class I–directed Cytotoxicity

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CTL recognize foreign antigens on the surface of target cells in association with MHC molecules. Alloreactive cells, which recognize MHC alloantigens as targets, exist as a significant fraction of the T cell repertoire in mature individuals and can be activated in vitro as a result of the allogeneic mixed lymphocyte reaction. MHC antigen variants distinguishable by CTL have been used to establish that changes in various positions of their amino acid sequence result in simultaneous alteration of epitopes recognized by both alloreactive and MHC-restricted CTL (1–5). In addition, some CTL show crossreactivity between class I allodeterminants and self–class I–restricted foreign determinants (6, 7). Thus, closely related epitopes on the MHC molecule could be involved in both allorecognition and MHC restriction. The existence of these crossreactions is in contrast with the virtual absence of CTL showing crossreactivity between class I and class II antigens. This lack of crossreactivity also holds at antibody level and is a well-known fact in HLA serology.

With a few exceptions, human CTL that recognize class I antigens express the CD8(T8) molecule on their surface, whereas those recognizing class II antigens express CD4(T4) (8). Anti-CD8 and anti-CD4 antibodies can prevent conjugate formation between target cells and their specific CTL (9–11). These observations prompted the assumption that CD8 and CD4 are involved in the recognition of nonpolymorphic sites on the class I and class II molecules, respectively (12). However, direct evidence for the interaction of CD8 or CD4 with MHC antigens has not been provided. A non–MHC-dependent regulatory role in T cell triggering has also been suggested for CD4 and CD8 (13, 14).

The present work shows the characterization of three alloreactive $CD8^+$ CTL clones that display simultaneous lytic ability for both HLA-$B27.1^+$ cells and HLA-$DR2^+$ targets expressing certain DR2-associated Dw determinants. For each of these clones, an anti-CD8 mAb inhibited class I–, but not class II–directed killing, whereas lysis of all target cells was inhibited by an anti-CD3 antibody.

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This is, to our knowledge, the first example of CTL clones displaying dual class I- and class II-directed alloreactivity. They were used to demonstrate a differential role of CD8 in the lytic mechanism of single clones, depending upon the MHC antigen class being recognized.

Materials and Methods

Cell Lines. Human lymphoblastoid cell lines (LCL)[1] as well as the erythroid tumour line K562 (used as an indicator of non-MHC-directed killer activity) were maintained in RPMI 1640 medium supplemented with 2 mM L glutamine, 10% heat inactivated FCS (Flow Laboratories, Inc., Middlesex, United Kingdom), and no antibiotics, at 37°C in a humidified atmosphere of 5% CO_2 in air. All LCL used as stimulators were mycoplasma free as assessed by seeding culture medium containing 2–4 × 10^6 cells in agar plates consisting of 1% Noble agar supplemented with PPLO broth, 1% yeast extract (Difco Laboratories, Inc., Detroit, MI) and 20% inactivated horse serum (Flow Laboratories, Inc.). The plates were incubated for 16 h in air at 37°C and for 6 d in a humidified atmosphere of 5% CO_2 in N_2 at 37°C. The presence of mycoplasmas was revealed with Dienes Stain (15). This test was routinely carried out once a month.

T Cell Cloning Procedures. Mixed lymphocyte cultures (MLC) and cloning were performed according to a method described elsewhere (16) with some modifications. In brief, 10^6 responder PBMC were stimulated with 5 × 10^4 irradiated (6,000 rad) LCL and 10^6 autologous PBMC as feeder cells. 10 d after stimulation, responding cells were either harvested and used for cloning (primary cloning) or restimulated with 5 × 10^4 LCL for a further period of 6 d, after which the cells were harvested and used for secondary cloning.

Responding cells from both primary and secondary cultures were cloned by limiting dilution procedures. For the primary cloning, cells were seeded at 16, 8, 4, 2, 1, and 0.5 cells per well in round-bottomed microwells containing 2 × 10^4 irradiated PBMC from the original responder donor and 2 × 10^3 LCL from the original stimulator in 40 μl final volume. For the secondary cloning, 10, 5, 2.5, 1.2, 0.6, and 0.3 cells per well were seeded. The cloning culture medium consisted of RPMI 1640 complemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin (all from Flow Laboratories, Inc.), 10% heat-inactivated pooled human serum from A+AB male donors, and 20 U/ml lectin-free IL-2 (Cellular Products Inc., Buffalo, NY). 7 d after cloning, all wells were restimulated with the same number of feeder cells and stimulators in a volume of 20 μl. After 10–14 d of incubation, 150 μl of complete medium were added to all proliferating clones from wells seeded with a number of cells that resulted in ≤63% growing wells. A prescreening for cytotoxicity of these clones against the original stimulator was performed. Cytotoxic clones were further expanded by seeding 1–2 × 10^4 cloned cells in round-bottomed wells containing 2–4 × 10^4 irradiated autologous PBMC and 2–4 × 10^3 irradiated LCL in a volume of 100 μl of cloning medium. 3 d later, 100 μl of cloning medium was added. The clones were restimulated once a week. Subcloning was carried out by limiting dilution of these clones exactly as described above.

Monoclonal Antibodies. The following mAbs were used: W6/32 (anti-HLA class I monomorphic determinant, reference 17) was a gift of J. L. Strominger, Harvard University, Boston, MA; ME1 (anti-HLA-B27 + B7 + B22, reference 18) was a gift from A. McMichael, Oxford University, Oxford, United Kingdom; EDU-1 (anti-HLA class II monomorphic determinant, reference 19) was a gift from R. Vilella, Hospital Clinico, Barcelona, Spain; L243 (anti-HLA-DR monomorphic determinant, reference 20), Genox 3.53 (anti-HLA-DQw1, reference 21) and B7.21 (anti-HLA-DP monomorphic determinant, reference 22) were kindly provided by C. Navarrete, from The London Hospital Medical College, London, United Kingdom; SPV-T3b (anti-CD3, reference 23), HP2/6 (anti-CD4, reference 24), and B9/4 (anti-CD8, reference 25), were provided by F. Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain.

[1] *Abbreviations used in this paper:* LCL, lymphoblastoid cell lines; MLC, mixed lymphocyte culture; SR, specific release.

FACS Analysis. Phenotyping of T cells was carried out by antibody binding followed by FACS analysis. T cell clones were cultured without feeder cells for a period of 10 d, after which 10^5 cells were incubated with 50 μl of the anti-CD3, -CD4, or -CD8 hybridoma supernatants and were stained with FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (Kallestad Laboratories, Inc., Austin, TX) at a final dilution of 1:50. FACS analysis was performed in an EPICS-C flow cytometer (Coulter Electronics Inc., Hialeah, FL) measuring the surface immunofluorescence of 5×10^3 viable cells.

Preparation of Target Cells for Cytotoxicity Assays. LCL were fed 24 h before 5×10^5 cells were labeled with 50 μCi of ^{51}Cr (Amersham Corp., Amersham, United Kingdom) for 1 h at 37°C in 100 μl FCS, with frequent stirring. The cells were then washed four times with warmed RPMI 1640 medium supplemented with 5% FCS. After resuspending in 2 ml of the same medium, the cells were counted and the radioactivity was measured in 50 μl of the cell suspension. They were then diluted to a concentration of $2–6 \times 10^4$ cells/ml, allowing a minimum of 800 cpm for a volume of 50 μl of target cell suspension. A minimum of 10^3 target cells were used for ^{51}Cr-release assays.

^{51}Cr-release Cytotoxicity Assay. CTL assays were performed in V-bottomed microtiter plates (Sterilin Ltd., Edtham, United Kingdom) according to a modification of a previously described method (16). Target cells ($1–2 \times 10^3$ ^{51}Cr-labeled LCL) were incubated with varying amounts of effector cells for 4 h at 37°C in a total volume of 150 μl. After centrifugation (5 min at $120 \times g$), 100-μl samples of cell-free supernatants were collected and their radioactivity was measured in a Packard Instrument Co., Inc. (Downers Grove, IL) gamma-counter. The percentage of specific ^{51}Cr-release (SR) was calculated as follows: $SR = 100 \times [(E - C)/(T - C)]$ where E is the cpm released by the target cells incubated with the effectors, C is the cpm released by the target cells incubated with medium alone, and T is the total cpm released by the target cells incubated with 100 μl of 0.1 M HCl. The C value was never >33% of the T value. The same procedure was used for prescreening of the clones as well as for testing of the subclones, except that 50-μl samples of the microwell-growing cell suspensions containing an unknown number of effector cells were incubated with 2×10^3 ^{51}Cr-labeled target cells in a final volume of 150 μl.

Inhibition of Cytotoxicity by mAbs. This was performed as described above, except that target (for anti-HLA mAbs) or effector (for anti-CD mAbs) cells were incubated with varying dilutions of the relevant mAb for 30 min at room temperature before the cytolytic assay, which was then carried out at a single E/T ratio. Anti-HLA mAbs were used at either 1:225 dilution of ascites fluid (W6/32, ME1, and EDU-1) or at 1:3 dilution of hybridoma supernatant (L243, Genox 3.53 and B7.21).

Cold-Target Inhibition Assay. Inhibition of cytotoxicity with unlabeled target cells was carried out as above except that varying amounts of the appropriate unlabeled cells were mixed with the ^{51}Cr-labeled targets before the addition of effector cells.

The percentage of inhibition (I) by both mAbs and cold target cells was calculated as follows: $I = 100 - [(\%\text{ lysis in the presence of inhibitor})/(\%\text{ lysis in the absence of inhibitor})] \times 100$.

Results

Isolation of CTL Clones. PBMC from donor PA (HLA-A24,33; B35,39; DRw6, DRw52; DQw1) and LCL LG15 (HLA-A32; B27.1; DR1; Dw1) were used respectively as responder and stimulator in primary and secondary MLC. The cells were cloned in the presence of LG15 in the primary cloning. For the secondary cloning, LCL R69 (HLA-A3,24; B7,27.1; DR3,5) was used as a stimulator cell. Limiting dilutions for both primary and secondary cloning fitted a linear regression line with $r = 0.99$. After prescreening for cytotoxicity against the original stimulator, positive clones were tested for anti-B27 activity by using mAb ME1 for cytotoxicity inhibition assays. 55 putative clones with apparent anti-B27 activity by these criteria were tested against a panel of LCL expressing three different HLA-B27 subtypes: LG15 (B27.1), R34 (B27.2), and Wewak I

(B27.3). Three of these clones (47, 23, and 27) were selected for their unusual pattern of reactivity since they lysed target cells LG15 and Wewak I, whereas R34 was not lysed. A fourth one, CTL 12, showed the same pattern of reactivity but was later excluded on the basis of subcloning and cold-target inhibition data that demonstrated the existence of an extra anti-HLA-B7 activity consistent with the presence of two separate clones in this CTL.

Clone 47 was obtained from those seeded at 1 cell per well in the primary cloning; clones 23 and 27 came from wells seeded with 0.3 cells in the secondary cloning. FACS analysis showed that clones 47, 23, and 27 have a homogeneous $CD3^+$, $CD8^+$, $CD4^-$ phenotype. All three clones were able to grow with similar efficiency when cultured in the presence of either LG15 or Wewak I as feeder cells for 1 mo. In both situations, the clones maintained an identical pattern of cytotoxicity against LG15, R34, and Wewak I target cells. No proliferation in the absence of exogenous IL-2 was observed in any of these clones using a panel of several stimulator LCL, including LG15 and Wewak I (data not shown). Upon subcloning, the same pattern of lysis was observed in all subclones obtained from clones 47, 23, and 27 (Table I).

Specificity of CTL Clones 47, 23, and 27: Panel Testing. The specificity of these clones was tested with a panel of target cells including HLA-typed LCL, PHA-stimulated blasts, and the K562 cell line (Table II). The optimal E/T ratio for all three clones was determined with LG15 and Wewak I in dose-response assays. These ratios were then used for all experiments and are also given in Table II.

Clones 47, 23, and 27 efficiently lysed B27.1 targets and were negative against B27.2, B27f, or B27.4 cells. This B27.1 specificity was confirmed by using PHA-stimulated blasts from $B27.1^+$ PBMC as targets, although the lysis of these blasts was less efficient than that of B27.1 LCL.

The panel data confirm the cytotoxicity of these clones against Wewak I. This cell line was more efficiently lysed than B27.1 targets. However, its lysis is not consistent with a B27.1/B27.3 crossreactivity since no significant lysis of other B27.3 target cells (SIA and ET) was observed. A reactivity against the other class I antigens of Wewak I (HLA-A11, A24, and Bw62) can probably be excluded since the clones did not lyse other target cells expressing these antigens (Table II). Clones 47, 23, and 27 lack non–MHC-directed cytotoxicity as assessed by negative lysis against K562. In addition, no cytotoxicity was observed against most $B27.1^-$ targets, with the exception of six DR2 homozygous target cells. Within these cells, DR2/Dw2 targets were killed with much less efficiency than those expressing the Dw specificities DB9 or 1859 (26, 27). Those $DR2^+$ cells that were DR2/Dw12 homozygous or DR2 heterozygous were not lysed. This reactivity could be responsible for the lysis of Wewak I, which is DR2 homozygous and whose Dw specificity is as yet not determined.

Inhibition of Cytotoxicity by Anti-HLA mAbs. To further analyze the specificity of these clones, the ability of several anti-HLA mAbs to inhibit the lysis of a panel of target cells was tested. Fig. 1 shows that the lysis of the LG15 cells (B27.1) was inhibited by mAbs W6/32, (anti–HLA class I monomorphic determinant) and ME1 (anti–HLA-B27 + B7 + Bw22). This lysis was not inhibited by mAb EDU-1 (anti–HLA class II monomorphic determinant). In contrast, the cytotoxicity against Wewak I was not inhibited by the two anti–class I mAbs but

TABLE I
Cytotoxicity of CTL 47, 23, and 27 Subclones

Clone number	r value	C*	Cells/well	Subclone number	Percent specific lysis of target cell		
					LG15	R69	Wewak I
47	0.93	34	12	1		26	20
				2		25	15
				3		8	8
			6	4		38	31
				5		11	5
				6		21	17
				7		13	18
			3	8		21	9
				9		23	12
23	0.99	22	20	1	79		77
				2	58		100
				3	41		95
				4	58		100
				5	51		81
				6	69		84
			10	7	39		94
				8	52		100
				9	43		100
				10	66		98
				11	72		100
				12	61		100
				13	28		74
			5	14	45		100
			1.2	15	45		60
				16	5		13
27	0.96	9	12	1		39	40
				2		46	48
				3		37	32
				4		39	29
				5		50	59
				6		52	44
			6	7		27	18
				8		25	19
				9		37	32
				10		29	26
				11		12	13
			1.5	12		52	61

Limiting dilutions for each clone fitted linear regression lines with r values as indicated.
* C is the number of cells seeded per well that gave a plating efficiency of 63%. E/T ratio for each subclone was unknown (see methods). For HLA typing of target cells, see Table II.

it was inhibited by EDU-1. Furthermore, the lysis of I2w9, PGF, and FJO (all $DR2^+$ targets) was also inhibited by EDU-1 and not by W6/32. These data confirm the apparent class II–associated specificity of these clones, additional to their anti-B27.1 specificity.

To clarify the nature of the anti–class II specificity of clones 47, 23, and 27, mAbs against HLA-DR, -DQ, and -DP molecules were used to inhibit the lysis

TABLE II

HLA Specificity of Clones 47, 23, and 27: Panel Study

Target cell	HLA						Clones*					
	A	B	Cw	DR	Dw	DQw	47	N	23	N	27	N
LG15	32	27.1	2	1	1	—	29	5	44	6	48	7
R69	3, 24	7, 27.1	—	3, 5	—	—	ND	—	39	2	53	3
LG2	2	27.1	—	1	1	—	72	1	65	1	67	2
R15	3	35, 27.1	—	—	—	—	85	1	77	1	ND	—
PHA blasts	2, 11	35, 27.1	1, 4	1	—	—	13	3	17	3	21	3
R34	2,24	18, 27.2	—	—	—	—	2	2	2	2	6	4
CHR	2, 3	35, 27.2	2, 4	1, 5	—	—	ND	—	0	1	1	2
R56	2, 11	14, 27.2	—	—	—	—	0	1	0	1	0	1
LH	24	8, 27f	1, 7	3, 4	—	—	4	1	0	3	2	3
We I‡	11, 24	62, 27.3	2, 4	2	—	—	77	5	65	6	78	8
SIA	2, 24	60, 27.3	4	3, 5	—	—	0	1	7	4	10	6
ET	2, 11	38, 27.3	—	4	—	—	1	1	0	1	2	1
PAR	11, 24	60, 27.4	—	2, 11	—	—	7	2	4	2	1	2
LIE§	2, 11	5, 27.4	—	12	—	—	6	1	3	3	4	2
HOM1	3	7	7	2	2	1	13	1	30	1	43	1
PGF	3	7	7	2	2	1	26	2	19	3	33	2
BBF	1	37	—	2	2	1	22	1	12	4	14	4
KT7	11, 24	52	—	2	12	1	0	1	0	2	0	1
TOK	24	52	—	2	12	1	0	1	2	2	1	1
I2w9	2, 26	57, 8w66	6	2	1859	—	94	1	68	3	75	1
FJO	2, 3	7, 16	—	2	DB9	1	73	2	68	3	75	1
BAS	2	51	—	2	DB9	3	61	1	58	1	83	1
AHA	2, 3	7, 57	6	2, 7	2, 11	1, 3	1	1	0	1	1	1
CHA	2, 3	7, 57	6	2, 7	2, 11	1, 3	0	1	0	2	2	1
We II§	11, 24	18, 39	6	2, 5	—	—	2	1	3	2	4	3
JY	2	7	—	4, 6	—	—	0	1	0	2	2	2
MEIN	1, 3	7, 57	6, 7	6, 7	—	—	0	1	0	1	0	2
S	24, 31	18, 60	3	4, 5	—	—	ND	—	ND	—	0	1
LB	28	60	3	6	—	—	0	1	2	2	8	2
B8N	3, 9	8, 15	3	3, 8	—	—	0	1	0	1	2	2
MCF	2	62	3	4	4	3	5	1	1	1	6	2
SWEIG	29	40	2	5	—	—	ND	—	1	2	0	3
K562¶							6	2	1	2	5	2

* Results are expressed as percent mean lysis from N experiments. E:T ratios were 10:1, 0.3:1 and 0.3:1 for clones #47, 23 and 27 respectively with all targets except R15 for which double ratios were used. ND = Not done.

‡ We, Wewak.

§ Spontaneous ^{51}Cr release (C) was 38–40% of the maximum release (T) for this cell line. See Materials and Methods.

¶ No HLA expressed.

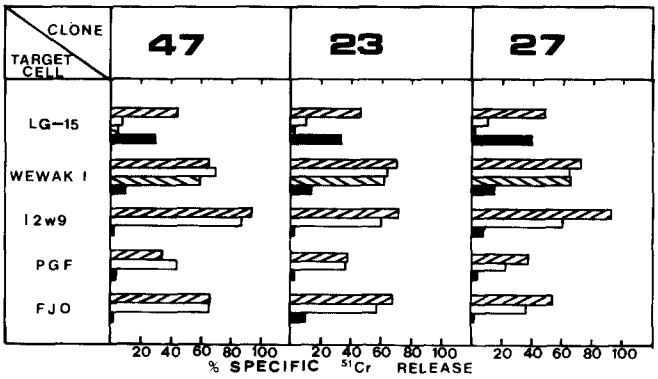

FIGURE 1. Inhibition of the cytotoxicity of clones 47, 23, and 27 by mAbs W6/32 (□), ME1 (▨), and EDU-1 (■). Cytotoxicity in the absence of mAbs is also shown (▨). The E/T ratio was 10:1, 0.3:1, and 0.3:1 with clones 47, 23, and 27, respectively for all target cells except for PGF, for which double E/T ratios were used. For complete HLA typing of LG15 ($B27.1^+$,$DR2^-$), Wewak I ($B27.3^+$,$DR2^+$), I2w9, PGF, and FJO ($B27^-$,$DR2^+$) see Table II.

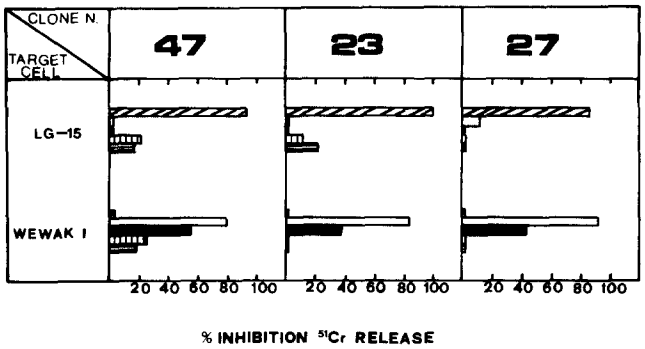

FIGURE 2. Inhibition of the cytotoxicity of clones # 47, 23 and 27 by mAbs W6/32 (▨), EDU-1 (□), L243 (■), Genox 3.53 (▩) and B7.21 (▣). E/T ratios were as specified in Fig. 1. Specific cytotoxicity of clones 47, 23, and 27 in the absence of mAbs was 22, 22, and 36%, respectively, for LG-15, and 69, 31, and 50%, respectively, for Wewak I.

of Wewak I. As shown in Fig. 2, only mAb L243 (anti-DR monomorphic determinant) inhibited the cytotoxicity, whereas Genox 3.53 (anti-DQw1) and B7.21 (anti-HLA-DP monomorphic determinant) showed no significant effect. The same results were obtained using target cells PGF and FJO (data not shown). These data strongly suggest that clones 47, 23, and 27 recognize a determinant associated with the DR2 molecule, and not a DP- or DQw1-related determinant.

Dual Reactivity of CTL Clones 47, 23, and 27: Cold-Target Inhibition Analysis. The experiments described above suggested a dual reactivity that makes clones 47, 23, and 27 capable of recognizing a class I determinant (B27.1) as well as a class II–associated determinant related to DR2. The HLA-DR2 targets I2w9, FJO, BAS, and Wewak I were the most efficiently killed, followed by the B27.1 targets and the DR2/Dw2 targets (Table II). To substantiate these differences in killing ability and to rule out the possibility that this unusual crossreaction was due to CTL 47, 23, and 27 not being true clones, cold-target inhibition assays were performed. Fig. 3 shows the results of such experiments, reciprocally

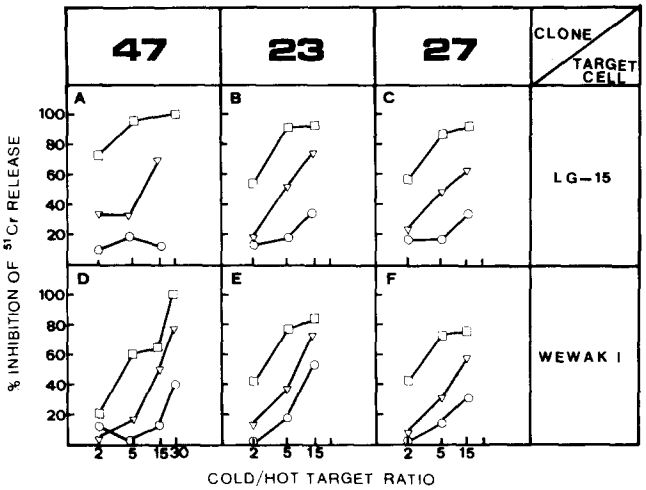

FIGURE 3. Cold-target inhibition analysis of the cytotoxicity of clones 47, 23, and 27. Hot-target cells were LG-15 (*A–C*) or Wewak I (*D–F*). Percent of specific ^{51}Cr release in the absence of competing cold cells was 27 (*A*), 53 (*B*), 56 (*C*), 80 (*D*), 74 (*E*), and 80 (*F*). Effector to hot target ratios were the same as those in Fig. 1. The following cold target cells were used: LG-15 (▽), Wewak I (□), and B8N (○). The latter was used as negative control. For HLA typing of these cells see Table II.

using LG15 and Wewak I as cold and hot targets: (*a*) the cytotoxicity against B27.1 (LG15) was strongly inhibited by Wewak I and not by an unrelated (B27.1$^-$, DR2$^-$) control target. This inhibition by Wewak I was higher than that obtained with LG15 itself; and (*b*) the cytotoxicity against Wewak I was inhibited by LG15, but at lower rate than with Wewak I itself. These results support the previous conclusions that CTL 47, 23, and 27 are clones and that they have a dual reactivity against B27.1 and DR2-associated determinants. They also confirm the panel data showing a greater efficiency of killing of Wewak I vs. B27.1 targets and suggest a higher avidity of these clones for Wewak I than for LG15.

In a similar series of experiments using clone 23 (Fig. 4), LG15 (B27.1), Wewak I (DR2/Dw "blank"), and PGF (DR2/Dw2) were used as hot targets and the same cells, as well as I2w9 (DR2/Dw 1859) and FJO (DR2/DwDB9), were used as cold targets. An unrelated LCL, JY (B27$^-$, DR2$^-$) was used as a control. Lysis of LG15 targets was strongly and equally blocked by Wewak I, I2w9, and FJO. Inhibition by PGF was similar to that obtained with LG15 itself and both were lower than that observed with the other DR2 targets. Cytotoxicity against Wewak I was slightly inhibited by LG15 and PGF and much more efficiently by I2w9 and FJO, at a rate as high as that observed with Wewak I itself. All B27.1$^+$ and DR2$^+$ cold targets inhibited the lysis of PGF. Analogous results were obtained using clone 47 (data not shown). These data confirm the monoclonal nature of CTL 23 and 47, as well as the heterogeneity of the DR2-related lysis by these clones.

Inhibition of Cytotoxicity by Antibodies against T Cell Surface Antigens: Role of CD3 and CD8. To investigate the role of the CD3 and CD8 molecules on this class I/class II crossreactive cytotoxicity, inhibition assays were performed using anti-CD3 and anti-CD8 mAbs to block the cytotoxicity of clones 47, 23, and 27. As

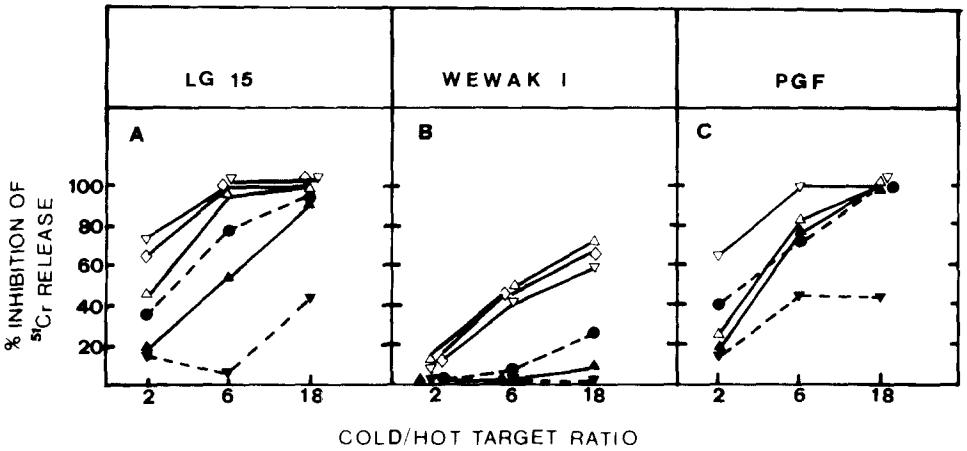

FIGURE 4. Cold-target inhibition analysis of the cytotoxicity of clone 23. Hot target cells were: (*A*) LG-15 (B27.1$^+$,DR2$^-$), (*B*) Wewak I (B27.3$^+$,DR2$^+$), and (*C*) PGF (B27$^-$, DR2/Dw2$^+$). Percent of specific ^{51}Cr release in the absence of competing cold cells was 40, 64, and 34%, respectively. Effector to hot target ratio was 0.3:1. The following cold target cells were used: I2w9 (—▽—), FJO (—◇—), Wewak I (—△—), PGF (—▲—), LG-15 (--●--), and JY (--▼--). The latter was used as negative control. For HLA typing of the cell lines see Table II.

shown in Fig. 5, the lysis of LG15 (B27.1) was clearly inhibited by anti-CD3 and anti-CD8 antibodies. The lysis of two different DR2$^+$ target cells, Wewak I and PGF, was strongly inhibited by mAb anti-CD3 irrespective of the significant differences in the susceptibility to lysis of these two cells. In contrast, anti-CD8 mAb not only did not inhibit such class II–directed killing but appeared to induce an enhancement of the cytotoxicity. A putative explanation for this enhancement could be the bridging of the effector and target cells by the anti-CD8 antibody through its heterologous binding to the Fc receptors on the target LCL, thus effectively increasing the avidity of the E/T interaction. This possibility was not experimentally addressed, because the anti-CD8 mAb used was IgG2b (25) and F(ab')$_2$ fragments for this subclass cannot be obtained (28). No effect of the anti-CD4 mAb was observed for any of the target cells. This antibody was used as a control to exclude the possibility that any residual CD4$^+$ cells not detected by FACS analysis could be contributing to the lysis of DR2$^+$ targets. These results strongly suggest that the CD8 molecule is involved in the class I–directed but not in the class II–directed cytotoxicity of these clones.

Discussion

In the present study, the specificity of three alloreactive CTL clones raised by stimulation of HLA-B27$^-$, DR2$^-$ responder cells with B27.1$^+$, DR2$^-$ cells has been analyzed. All three clones showed an identical pattern of reactivity in that they specifically lysed target cells expressing either HLA-B27.1 or a subset of HLA-DR2, but the lytic capacity of CTL 47 was significantly lower, possibly reflecting less avidity for the corresponding targets. The monoclonal character of these clones was established on the following bases: (*a*) they were phenotypically homogeneous CD3$^+$, CD8$^+$, CD4$^-$; (*b*) their specificity was homogeneous

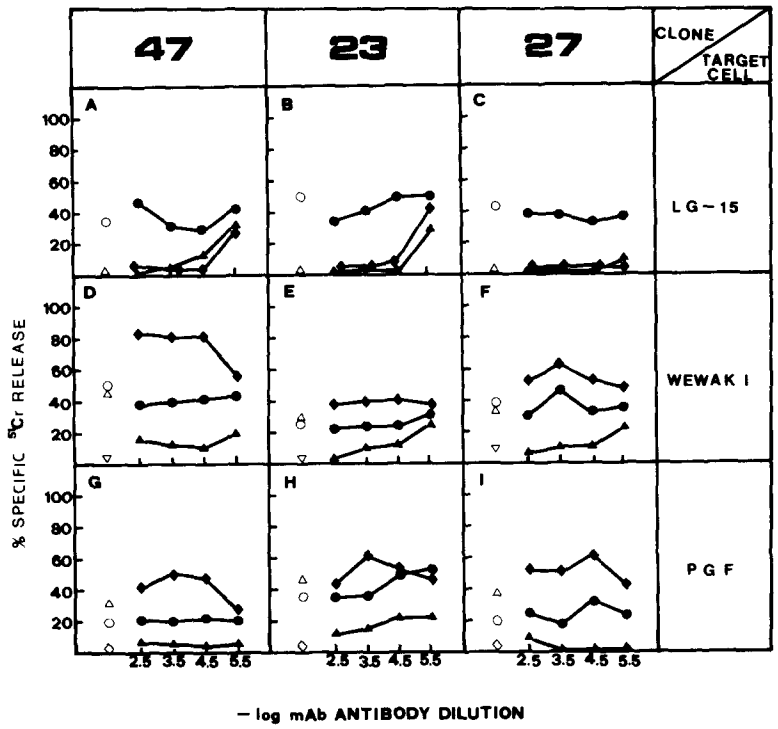

FIGURE 5. Inhibition of the cytotoxicity of clones 47, 23, and 27 against LG-15 (*A–C*), Wewak I (*D–F*), or PGF (*G–I*) by the anti-CD3 mAb SPV-T3b (▲), the anti-CD8 mAb B9/4 (♦) and the anti-CD4 mAb HP2/6 (●). Inhibition of cytotoxicity by the anti-HLA mAbs W6/32 (△), EDU-1 (▽) and L243 (◇) was used as control. The specific ^{51}Cr release in the absence of antibody (○) is also shown. For clone 47, E/T ratios were 10:1 with LG-15 and PGF, and 5:1 with Wewak I. For clones 23 and 27, E/T ratios were 0.3:1 with LG-15 and PGF and 0.15:1 with Wewak I. For HLA typing of target cells see Table II.

upon subcloning; and (*c*) HLA-B27.1$^+$ targets and HLA-DR2$^+$ targets reciprocally inhibited each other in cold-target inhibition assays. That these clones were alloreactive for both HLA-B27.1 and HLA-DR2, as opposed to being allo–class II–specific, self–class I–restricted or vice versa, was clear from a number of criteria. First, HLA-B27.1 was absent in the responder cells and HLA-DR2 in both the responder and the stimulator cells. Second, lysis of DR2$^+$ targets was accomplished independently of whether they shared class I antigens with their effector cells. Third, lysis of HLA-B27.1$^+$ targets by all three clones was inhibited by anti–class I but not by anti–class II antibodies. In contrast, lysis of HLA-DR2 target cells was inhibited by anti–class II but not by anti–class I antibodies. In addition, all three clones lysed B27.1$^+$, DR2$^-$ PHA-stimulated blasts, and at least CTL 23 also lysed DR2$^+$, B27.1$^-$ blasts (not shown), thus excluding the possibility of these clones being EBV-specific, class I or class II restricted. Taken together, these results indicate that CTL clones 47, 23, and 27 display dual alloreactivity for both HLA-B27.1 and a subset of HLA-DR2.

Neither the possibility of two separate receptors nor that of one receptor with two separate binding sites being expressed on these CTL clones can be excluded from the present data. However, no examples of either situation have been

demonstrated so far. Thus, we consider that the simplest explanation for this dual reactivity is that the clones recognize a crossreactive determinant shared between HLA-B27.1 and a subset of HLA-DR2. Some experimental grounds for this alternative are provided by findings (29–31) suggesting that both class I and class II antigen recognition may be mediated by the same set of T cell receptor V genes. Therefore, with the above mentioned alternatives in mind, further discussion will be based on the assumption that each of these clones express a single antigen receptor with a single binding site.

Some insight into the nature of the putative determinant recognized by these CTL clones can be obtained from their highly selective pattern of reactivity for both the HLA-B27.1 subtype and certain HLA-DR2 antigens. Molecular analyses of HLA-B27 antigens strongly suggest that the epitope recognized by clones 47, 23, and 27 on HLA-B27.1 includes at least the Asp residue at position 77, since this residue is the only one that is unique to B27.1 among all B27 subtypes whose structure has been examined (3, 4, 32, and our unpublished results).

The HLA-DR2 antigen displays a remarkable polymorphism that can be detected by cellular typing and includes at least the Dw2, Dw12, FJO/DB9, and the 12w9/1859 specificities (26, 27). Biochemical and DNA sequence analyses have shown that this polymorphism is associated to heterogeneity in two DR β chains expressed on HLA-DR2 homozygous cell lines, as well as with polymorphism in the α and β chains of DQw1 (33–37). The epitope recognized by CTL clones 47, 23, and 27 closely correlates with the DR2-associated Dw polymorphism, since Dw12 targets are not recognized by these clones and since 12w9, FJO/DB9, and Dw2 targets are lysed with different degrees of efficiency. The lack of killing of HLA-DR2 heterozygous target cells (Table II) might be due to their lower expression of DR2. In addition, at least two of these cells were Dw2, for which the lytic capacity of these clones is much lower. Since the DR2-directed killing can be significantly inhibited by an anti-DR but not by an anti-DQw1 mAb, it is likely that the putative crossreactive epitope recognized by these CTL clones is located on the DR2 β chains and is related to the Dw-associated polymorphism of these polypeptides. This is further supported by the fact that the $DR2^+$, $DQw1^-$ cell line BAS was killed with comparable efficiency to other $DR2^+$, $DQw1^+$ targets (Table II).

Whatever the molecular bases for the crossreaction between HLA-B27.1 and various DR2-associated Dw specificities, its detection suggests that some structural feature of the HLA-B27.1 molecule may mimic a class II determinant that is closely related or identical to those that can induce T cell proliferation. The biological implications of this phenomenon are unknown. Crossreactions between class I and class II antigens are virtually absent in HLA serology. Thus, it would be interesting to analyze whether the type of crossreactivity found in CTL clones 47, 23, and 27 reflects a unique property of HLA-B27 or a more general one of the HLA class I antigens, so that the distinction between class I and class II determinants recognized by T cells could be somewhat les strict than for serologic determinants.

The availability of individual $CD3^+$,$CD8^+$,$CD4^-$ CTL clones with simultaneous class I– and class II–directed lytic ability allowed us to examine the role of CD8 in the lysis of targets bearing the relevant class I or class II molecules by the

same CTL. The involvement of the T cell receptor complex in both the B27.1-directed and DR2-directed killing by these clones was strongly supported by the fact that lysis of both $B27.1^+$ or $DR2^+$ targets was inhibited by an anti-CD3 mAb. In contrast, an anti-CD8 mAb selectively blocked the lysis of $B27.1^+$ targets but not that of $DR2^+$ targets. Furthermore, the lack of inhibition of the DR2 targets was independent of the different lytic efficiency of these clones for targets expressing the various DR2-associated Dw specificities. CTL clones are heterogeneous in their susceptibility to inhibition by anti-CD3 and anti-CD8 antibodies (38), and this effect has been related to the avidity of the E/T interaction (16, 39, 40). However, it is very unlikely that differences in the relative avidity of clones 47, 23, and 27 for B27.1 vs. DR2 targets are responsible for the differential blocking of anti-CD8 because the PGF(DR2/Dw2) and LG-15(B27.1) cells reciprocally inhibited each other in cold-target inhibition experiments with similar efficiency (Fig. 4). Furthermore, as mentioned above, anti-CD3 mAb inhibited both B27.1 and DR2 targets. A similar lack of inhibition by anti-CD8 (or anti-Lyt-2,3) mAb has been observed with some $CD8^+$ or Lyt-$2,3^+$ anti–class II CTL (41, 42). Most probably, the selective inhibition of the class I–directed lysis by clones 47, 23, and 27 reflects a different role of the CD8 molecule depending on the MHC antigen class being recognized on the target cell.

It is generally assumed that CD8 contributes to increasing the overall avidity of the E/T interaction either by interacting with nonpolymorphic regions of the class I molecules (12) or by otherwise stabilizing the binding of the CTL receptor to the corresponding antigen (39). The differential inhibition of the class I– but not the class II–directed cytotoxicity of clones 47, 23, and 27 by the anti-CD8 mAb is not consistent with a simple additive contribution of the CD8 interaction with class I antigen to the avidity of the CTL for the target cell because all targets used in this study simultaneously expressed class I and class II antigens. The present results can be coherently interpreted by postulating that stabilization of the T cell receptor–MHC antigen interaction by CD8 is accomplished through the binding of this molecule to a different epitope on the same class I molecule bound by the antigen receptor. Anti-CD8 mAbs would inhibit lysis by disrupting this ternary complex. In this model, a physical association of the T cell receptor and the CD8 molecule is not required in the absence of an MHC antigen on the target cell to which both the receptor and CD8 can bind. Since CD8 would not bind to class II antigens, the anti-CD8 antibody would not have any disruptive effect in the class II–directed lysis of target cells by these CTL clones and would not inhibit lysis in those cases. This interpretation is analogous to the role postulated recently (43) for L3T4 to explain the behaviour of two unusual Lyt-2^+, $L3T4^+$ hapten-specific, class II MHC–restricted T cell clones, whose antigen-induced lymphokine production was inhibited by anti-L3T4 but not by anti-Lyt-2 antibodies.

A regulatory role in CTL triggering has been postulated for CD8 (14). This is not incompatible with an additional role of CD8 in stabilizing the E/T interaction, such as the one proposed above. But an exclusive regulatory role of CD8 appears doubtful on the basis of our results because it would mean that the negative signal generated upon anti-CD8 antibody binding was effective only for

the class I–dependent lysis but not when the same clone was recognizing a class II antigen.

In view of the complexity of the molecular events leading to CTL recognition and lysis and the difficulties in designing experimental systems for their direct analysis, the availability of unusual CTL clones, such as those discussed here, can provide unique tools for examining the function of multiple cell surface molecules involved.

Summary

HLA-B27$^-$ responder cells were stimulated in vitro with HLA-B27.1$^+$ lymphoblastoid cell lines, and alloreactive CTL clones were obtained by limiting dilution. Three of these clones specifically lysed B27.1$^+$ targets. In addition, they also lysed homozygous DR2 targets with various degrees of efficiency, depending on the Dw specificity of the target cell. All three clones possessed a homogeneous CD3$^+$,CD8$^+$,CD4$^-$ phenotype and were also homogeneous upon subcloning. Cold-target inhibition analyses showed mutual inhibition of B27.1 target lysis by DR2 targets and vice versa. Lysis of B27.1 targets was selectively inhibited by anti–class I mAbs. In contrast, lysis of DR2 targets was inhibited only by anti–class II and anti-DR monomorphic antibodies, but not by anti–class I, anti-DQw1, or anti-DP antibodies. The results indicate that these clones display dual recognition for HLA-B27.1 and for HLA-DR2 and suggest that HLA-B27.1 may share at least one epitope that is closely related to some stimulatory Dw determinants present on the HLA-DR2 antigens.

Lysis of both B27$^+$ and DR$^+$ targets was inhibited by an anti-CD3 mAb. In contrast, an anti-CD8 antibody selectively inhibited the B27- but not the DR2-directed killing by these clones. The data support a stabilizing role of CD8 through its binding to the same class I (but not class II) molecule on the target cell bound by the T cell antigen receptor.

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Note added in proof: Results analogous to those described in this paper have recently been reported in the murine system (Schilham, R. Lang, R. Benner, R. Zinkernagel, and H. Hengardner. 1986. Studies of an alloreactive CTL clone specific for H-2D^b that crossreacts with I-E^k. *J. Immunol.* 137: 2748.).

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