Immunology 1988 **64** 87–92

Lysis of CD3 hybridoma targets by cloned human CD4 lymphocytes

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Accepted for publication 11 January 1988

SUMMARY

UCHT1, a CD3-producing hybridoma, is lysed by cloned human T cells of both CD4 and CD8 phenotypes. The lysis is specific for the CD3 receptor and is mediated by 90% or more of all T cells from blood, without evidence for a subset of CD4 T cells incapable of cytotoxicity. CD4 clones characterized as 'helper' cells by *in vitro* help for specific antibody production by B cells also lysed CD3 targets. Varicella zoster virus (VZV) super-infected B lymphoblasts were lysed by three of six VZV-specific T-cell clones and 1Cl-specific T-cell clones lysed 1Cl hybridoma targets. Resting B cells (from tonsil) coated with CD3 antibody were lysed by CD4 clones. The data suggest that the majority of clonable $CD4^+$ T cells lyse target cells recognized via CD3 or through the antigen receptor.

INTRODUCTION

T cells are commonly referred to as cytotoxic or helper or suppressor, but uncertainty remains as to whether these functions are really restricted to different subsets of T cells. Although CD4 T cells were originally characterized as 'helper' because their presence was necessary for B cells to respond to pokeweed mitogen (Thomas, Rogozinski & Chess, 1983), both suppressor (Jacoby & Oldstone, 1984) and cytotoxic (Krensky *et al.*, 1982) CD4 T cells have been reported subsequently. Antigen recognition restricted by class II MHC molecules is a common property of CD4 T cells (Meuer, Schlossman & Reinherz, 1982), but class II-restricted cytotoxicity by these cells is reported increasingly (Jacobson *et al.*, 1984; Yasukawa & Zarling, 1984; Hayward *et al.*, 1986b). These observations raise the possibility that cytotoxicity is a property of all T cells following appropriate recognition of a target. In order to test this hypothesis directly, we sorted lymphocytes into CD4 and CD8 subsets and then stimulated these populations with CD3 antibody under limiting-dilution conditions. The clones obtained were then tested for their ability to lyse the CD3-making hybridoma, UCHT1 (Beverley & Callard, 1981). To parallel these results in a system requiring target recognition through the T-cell receptor, two additional stimuli were tested: Varicella zoster virus antigen and 1Cl, a hybridoma which makes an antibody to a T-cell receptor beta-chain family (Boylston & May, 1986). The susceptibility of resting B cells to lysis was tested by re-targeting T-cell clones with a CD3–anti-HLA class I antibody complex (Perez *et al.*, 1985).

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MATERIALS AND METHODS

Preparation of lymphocytes and antigen-specific T-cell clones

Blood from normal volunteers was defibrinated and centrifuged for 10 min at 700 *g* so that the serum could be aspirated. The cells were resuspended in Hanks' balanced salt solution (ICRF media preparation) buffered with HEPES (Gibco, Paisley, Renfrewshire) (HBSS) and centrifuged on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) (SG 1·077, 750 *g* for 20 min). Mononuclear cells (MNC) at the interface were harvested, washed twice in HBSS and resuspended in RPMI-1640 (Gibco) at 10^6 cells/ml. Varicella zoster virus (VZV)-specific T-cell clones were prepared as described elsewhere (Hayward *et al.*, 1986b). Briefly, 10^7 MNC were cultured with 5×10^5 glutaraldehyde-fixed VZV-infected fibroblasts in Falcon 25 cm^2 flasks (Becton-Dickinson Labware, Lincoln Park, NJ; cat. no. 3013) for 7 days then 10 μ/ml IL-2 (ala-125, batch 308; Amgen, Thousand Oaks, CA) were added. Seven days later the cells were centrifuged on Ficoll–Hypaque (750 *g*, 10 min) and the viable cells at the interface recovered and washed. These cells were then cultured at 100, 50, 25 and 12·5 cells per well in Linbro microwells (Flow Laboratories, Irvine, Ayrshire) (76-018-04) with 5×10^4 irradiated autologous MNC and VZV antigen. After 7 days, 0·1 ml of medium containing 2 U IL-2 were added to each well. Clones visible to the naked eye were harvested 10 days later and expanded on irradiated autologous MNC and antigen and 10 μ/ml IL-2 prior to testing for VZV specificity by proliferation (Hayward *et al.*, 1986b). Specific clones were re-cloned at 1 cell per well as above.

Preparation of clones from blood MNC

Blood MNC were stained with FITC-conjugated CD4 (Becton-Dickinson, Monoclonal Antibodies, Mountain View, CA; cat. no. 7413) or CD8 (cat. no. 7313) according to the makers

instructions (Becton-Dickinson, 1985), washed twice in HBSS and sorted for FITC-positive cells on a FACS I gating for cell size on forward light scatter. The sorted cells were collected into RPMI-1640 with 10% fetal calf serum (FCS) (Gibco) and were counted directly in suspension, without centrifugation. Counts of cells recovered were 65–75% of the counts recorded, as sorted by the FACS. The sorted cells were diluted further with medium containing 10^5 irradiated autologous MNC and 10^5 irradiated UCHT1 (ICRF Human Tumour Immunology Group) cells and 10 μ/ml IL-2. The sorted cells were plated in 0·1 ml volumes at 0·25, 0·5, 1, 2, 4 and 8 cells per well in Linbro microwell plates. After 7 and 14 days of culture the wells were fed with 0·1 ml medium containing 10 U/ml IL-2.

Proliferation assays

Proliferation by antigen-specific T-cell clones was measured by uptake of [^{3}H] thymidine [^{3}H]TdR (TRK 686, Amersham International, Amersham, Bucks, 1 μCi/well) over a 4-hr period. The cells were harvested onto glass fibre and counted in a liquid scintillation counter. Proliferation by UCHT1-stimulated clones was measured by pulsing wells with 0·05 μCi of ^{125}I-UdR (IM 355, Amersham International) over a 18-hr period. These cells were harvested onto glass fibre and counted in a gamma counter. Counts of ^{125}I-UdR in the presence of ^{51}Cr were corrected for cross-talk.

Cytotoxicity assays

Hybridoma targets were labelled with 100 μCi ^{51}Cr (sodium chromate CJS 1; Amersham International) per 10^6 cells for 40 min. The cells were centrifuged on Ficoll–Hypaque (SG 1·088, 10 min, 20°). Cells from the interface were washed three times and adjusted to 25,000 cells/ml in RPMI-1640 with 10% FCS. Cytotoxicity assays were in V-bottomed plates containing 0·1 ml targets and 0·1 ml effectors at E:T ratios stated. The plates were centrifuged briefly (200 ***g***, 4 min) before incubation for 4 hr at 37°. Where proliferation and cytotoxicity assays were performed on the same well of limiting-dilution cultures the ^{125}I-UdR was added overnight and the ^{51}Cr-labelled targets were added with mixing the following morning.

Lysis of uninfected and VZV-super-infected, autologous or unrelated, EBV-transformed lymphoblasts was measured by ^{51}Cr release (Hayward *et al.*, 1986b). PHA-induced lysis was tested with uninfected autologous B lymphoblasts as targets and PHA (Wellcome Reagents, Beckenham, Kent, HA 17) at 1μg/ml. Specific lysis was measured as

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

Test for antigen-specific help for B cells

VZV-specific T-cell clones were washed free of IL-2 and adjusted to 2×10^5 cells/ml in RPMI with 10% FCS. T-depleted cells were prepared from blood MNC by rosetting with AET-treated sheep erythrocytes (S-2-aminoethylisothiouronium bromide hydrobromide; Aldrich Chemical Co., Gillingham, Dorset) (Kaplan & Clark, 1974) and adjusted to 10^6 per ml. In order to test for help, 2×10^5 T cells were cultured with 2×10^6 T-depleted cells with or without 10^4 glutaraldehyde-fixed VZV-infected fibroblasts in Costar (Cambridge, MA) 24-well plates (cat. no. 3524). The medium was replaced with fresh RPMI-1640 with 10% FCS after 3 days and the cultures terminated after 10 days, when the medium was harvested. This was tested undiluted for anti-VZV antibody by ELISA as previously described (Hayward, Herberger & Corey, 1986a). Parallel control cultures included unseparated MNC, T-depleted cells without T cells and T clones without non-T cells. To control for the specificity of the ELISA, culture supernatants were also tested on control antigen preparation. These negative controls gave ODs of $< 0{\cdot}05$.

Preparation of hybrid antibodies

CD3 (from UCHT1) and anti-HLA class I framework (from W6/32) were precipitated from ascites with 50% ammonium sulphate and dialysed against phosphate-buffered saline (PBS) (ICRF media preparation). Two milligrams of each antibody were coupled with SPDP [*N*-hydroxysuccinimidyl3-(2-pyridyldithio) propionate; Pharmacia, Uppsala, Sweden] by the method of Carlsson, Drevin & Axen (1978). SPDD was added to 50 μM final concentration for 30 min at room temperature, after which the protein was dialysed against three changes of PBS for 24 hr. The CD3 antibody was then thiolated with dithiothreitol and again dialysed before mixing with the anti-HLA antibody overnight at 4°. The mixture was separated on a small G50 column in PBS and concentrated to 2 mg/ml.

Preparation of targets with hybrid antibodies

Cells teased from tonsils were adjusted to 5×10^6 per ml and depleted of T cells by rosetting (Kaplan & Clark, 1974) before being layered onto a 40–65% Percoll gradient (5% steps) and centrifuged (10 min, 800 ***g***, 4°). Cells at the 55% interface were recovered and washed for use as targets. Hybrid antibody (200 μg/10^6 cells) was added to targets at the same time as the ^{51}Cr.

Hybridoma cell lines

Hybridomas used in these experiments, and their antibodies were as follows: UCHT1 (CD3); UCHT2 (CD5); MHM6 (CD23, generously provided by Professor A. J. McMichael, John Radcliffe Hospital, Oxford); 1Cl = anti-beta family chain of T-cell antigen receptor generously provided by Dr A. Boylston (St Mary's Hospital Medical School).

RESULTS

Specificity of CD3 hybridoma lysis

Initial studies confirmed that the CD3 hybridoma targets, but not the CD5 or CD23 hybridoma targets, were lysed by cloned CD4 or CD8 T cells (Table 1). In the 4-hr assay used, lysis was specific for CD3 hybridoma in that bystander CD5 or MHM6 targets were not lysed, even in the presence of CD3 hybridoma cells. The ability of CD3 and LFA1 antibodies, but not anti-HLA antibody, to interfere with lysis is consistent with the results of others (Hoffman *et al.*, 1985), as is restriction of PHA-induced lysis to the $CD8^+$ subset (Reinherz & Schlossman, 1980).

CD3 hybridoma targets are lysed by CD4 and CD8 T-cell clones

Sorted CD4 and CD8 T cells were cultured at 0·25, 1, 2 and 4 cells per well with autologous irradiated MNC and CD3 hybridoma cells and IL-2. Controls wells contained irradiated

Table 1. Specificity of lysis of UCHT1 by cloned CD4 and CD8 cells*

Effector clone	Hot target	Cold target	Added antibody/PHA	% lysis at E:T ratio: 20:1	10:1	5:1
CD4	UCHT1	None	None	92	72	55
CD4	UCHT1	UCHT1	None	44	22	18
CD4	UCHT1	UCHT2	None	93	73	54
CD4	UCHT1	MHM6	None	93	78	49
CD4	UCHT1	None	W6/32	87	70	48
CD4	UCHT1	None	LFA1	55	47	19
CD4	UCHT1	None	CD3	47	30	12
CD4	UCHT2	None	None	5	1	1
CD4	UCHT2	UCHT1	None	2	2	1
CD4	UCHT2	None	CD3	1	1	2
CD4	MHM6	None	None	3	3	2
CD4	B blasts	None	None	1	1	1
CD4	B blasts	None	PHA	1	2	1
CD8	UCHT1	None	None	90	73	66
CD8	UCHT1	UCHT1	None	40	34	22
CD8	UCHT1	UCHT2	None	91	75	60
CD8	UCHT1	MHM6	None	91	72	61
CD8	UCHT1	None	W6/32	88	68	58
CD8	UCHT1	None	LFA1	47	28	18
CD8	UCHT1	None	CD3	41	29	19
CD8	UCHT2	None	None	13	6	5
CD8	UCHT2	UCHT1	None	12	6	6
CD8	UCHT2	None	CD3	8	7	4
CD8	MHM6	None	None	6	5	6
CD8	B blasts	None	None	2	3	1
CD8	B blasts	None	PHA	51	32	18

* Effectors, 50,000, were cultured with 5000 hot targets and, where indicated, 50,000 cold targets. Competing antibodies were added at 25 μg/ml. Results shown are from representative CD4 and CD8 clones, similar results were obtained with a further six clones.

cells and IL-2 but no sorted T cells. After 18 days, when clones were visible to the naked eye, all the culture wells were tested for proliferation (as ^{125}I-UdR uptake) and for cytotoxicity (by ^{51}Cr-release from CD3 targets). In order to analyse the data the mean $\pm$ 3 SD of ^{51}Cr release and $\log_{10}$ ^{125}I-UdR uptake in the controls were determined first. Responses in the limiting-dilution cultures in excess of these levels were indicative of proliferation or cytotoxicity. Data from wells seeded at 1 and 0·25 cells per well from four experiments are pooled in Table 2 and they indicate that 95 of 103 CD4 (92%) and 139 of 143 CD8 (97%) clones were cytotoxic. Visual inspection confirmed that no clones were present in the wells negative for both proliferation and cytotoxicity. In addition to this contingency analysis, $\log_{10}$ ^{125}I-UdR uptake of wells was plotted against ^{51}Cr release. The results of a representative experiment (Fig. 1) show that, for both CD4 and CD8 clones, there is a positive correlation between proliferation and cytotoxicity. This correlation is significant when tested by regression ($r = 0{\cdot}61$, F-test $= 43{\cdot}35$, $P < 0{\cdot}0001$ for CD4 clones; $r = 0{\cdot}65$, F test $= 53{\cdot}0$, $P < 0{\cdot}0001$ for CD8 clones).

Cloning efficiencies were calculated for each experiment and ranged from 33% to 87%. Unsorted T cells separated from blood MNC by E-rosetting had a cloning efficiency in three experiments under the same culture conditions of 70–90%. One-hundred and five proliferative wells were obtained from wells seeded at 1 cell per well or less, of which 103 (98%) were cytotoxic for UCHT1 cells. Consequently, although our data suggest that every T cell cloned was capable of lysing CD3$^+$ targets, they do not exclude the possible existence of a minor 'helper' subset which is not stimulated to proliferate with CD3 antibody and which is incapable of cytotoxicity. To look for this subset we next tested 'helper' T-cell clones with specificity for viral antigens.

Table 2. Correlation between cytotoxicity and proliferation by 2 × 2 table*

Proliferation	CD4 wells cytotoxicity: Positive	CD4 wells cytotoxicity: Negative	CD8 wells cytotoxicity: Positive	CD8 wells cytotoxicity: Negative
Positive	87	8	120	3
Negative	8	279	19	240

* Data derived from wells seeded with 0·25 or 1 cell in four separate experiments.

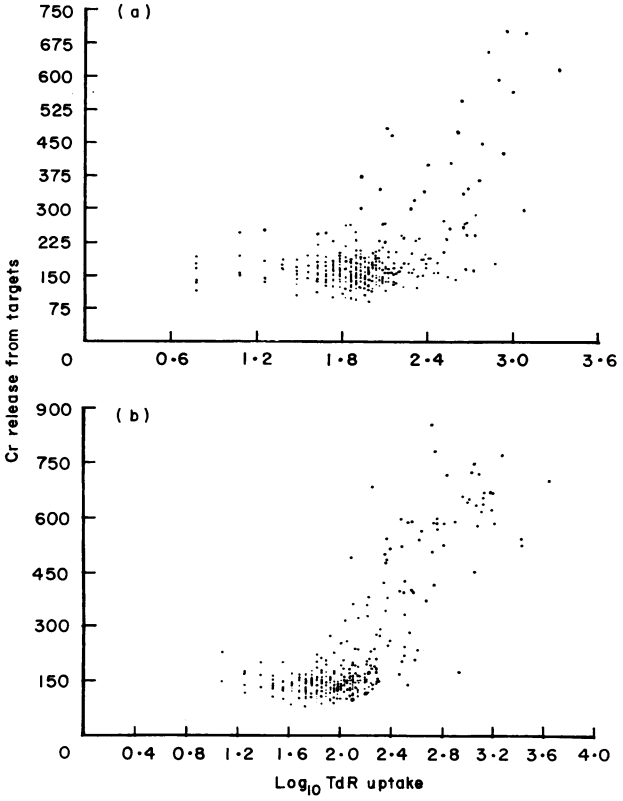

Figure 1. Histogram of proliferation (as $\log_{10}$ ^{125}I-UdR uptake) against cytotoxicity (^{51}Cr release from targets) for CD4 (a) and CD8 (b) T cells cultured at limiting dilution. Targets = UCHT1 cells. Spontaneous release counts = 190 $\pm$ 16, maximum release = 840 $\pm$ 68.

Table 3. T-cell clones with helper activity for B cells lyse CD3 hybridoma targets*

Clone	Specificity	B-cell help	Specific cytotoxicity	% lysis of: CD3	CD5
HA 1.7	Flu	ND†	–	48	1
12	VZV	0·32	+	48	2
15	VZV	0·47	–	57	2
A7	VZV	0·64	+	71	1
11	VZV	0·11	–	54	1
17	VZV	0·11	+	57	2
21	VZV	0·11	+	74	2

* E:T ratio 10:1. T-cell clones were tested at end of feeding cycle.

† Helper activity for HA 1.7 B cells documented by Lamb & Green (1983). Helper activity of VZV-specific T clones presented as OD for IgG antibody to VZV in culture supernatants of clones with antigen and autologous B cells (see the Materials and Methods).

Table 4. Correlation between 1Cl-specific cytotoxicity by 2 × 2 table

Proliferation	CD4 wells cytotoxicity: Positive	CD4 wells cytotoxicity: Negative	CD8 wells cytotoxicity: Positive	CD8 wells cytotoxicity: Negative
Positive	3	1	6	0
Negative	0	124	0	122

Table 5. Lysis of Percoll fraction of tonsil lymphocytes containing small B cells*

Experiment no.	Specific lysis of targets coated with: Control	Hybrid antibody
1	1	61
2	1	54
3	4	65
4	1	55
5	2	46

* Effectors in these cultures were five $CD4^+$ clones of VZV-specific T cells. E:T ratio = 10:1. Control targets were small B cells without pre-incubation in hybrid antibody.

CD3 hybridoma targets are lysed by 'helper' T-cell clones

The results of the limiting-dilution cultures suggested that cytotoxicity was a property of most, and perhaps all, clonable T cells. In order to determine whether T-cell clones which helped B cells for antibody production also lysed CD3 hybridoma targets, we selected six VZV-specific T clones and HA 1.7 (an influenza virus haemagglutinin-specific clone with helper activity, demonstrated by Lamb & Green, 1983). VZV-specific clones were tested for helper activity for autologous B cells and three were found which provided help for IgG antibody production (ELISA OD $>0{\cdot}16$). The results (Table 3) indicate that each clone lysed the CD3 targets.

These results suggested that T-cell clones with demonstrable helper activity for B cells could lyse targets recognized through cell surface CD3. The fact that four of the six VZV-specific T-cell clones also lysed VZV super-infected autologous B lymphoblasts suggested that lysis might also proceed through recognition via the T-cell antigen receptor. In order to test this possibility we next tested clones with specificity for 1Cl (a hybridoma with specificity for a beta chain family; Boylston & May, 1986).

1Cl hybridoma targets are lysed by 1Cl-specific T-cell clones

1Cl-specific T-cell clones were prepared by culturing sorted CD4 and CD8 T cells with 10^4 each of irradiated hybridoma cells and autologous MNC per well. Because 1Cl-positive cells account for only 5% of circulating T cells, the culture wells were seeded with 20–80 CD4 or CD8 cells. Clones were picked after 14 days culture and expended on irradiated 1Cl hybridoma cells for 7 days before immunofluorescence staining for 1Cl. Parallel testing of cultures of T cells positive for the surface 1Cl antigen for proliferation and cytotoxicity (for 1Cl targets) showed a strong positive correlation (Table 4). Most of the clones obtained were negative for 1Cl binding: these did not lyse 1Cl targets though they did lyse UCHT1 targets (data not shown).

Helper T-cell clones can lyse small B lymphocytes

The ability of T cells with helper activity to lyse CD3 hybridoma targets, and autologous antigen-specific B lymphoblastoid targets, raised the possibility that resting B lymphocytes might also be killed. In order to test this, small B lymphocytes were purified from suspensions of tonsil lymphocytes by E-rosette depletion and Percoll density gradient centrifugation. Over 85% of cells from the 55% Percoll interface expressed the B-cell surface antigen, CD20, by immunofluorescence. When these cells were sensitized with an anti-HLA-CD3 hybrid antibody they were lysed by all T clones tested (Table 5).

DISCUSSION

It is clear that T cells show functional restrictions in their responses to antigen *in vivo* and *in vitro*. For example, $CD4^+$ cells are required for antibody responses to virus antigens (Nash *et al.*, 1987) and $CD8^+$ cells are unable to substitute for $CD4^+$ cells for a response by human B cells to pokeweed mitogen (Thomas *et al.*, 1983, Reinherz & Schlossman, 1980). CD8 cells lyse influenza virus-infected target cells (Dongworth & McMichael, 1984) and this subset of lymphocytes is commonly described as cytotoxic/suppressor. However, target cell lysis by

$CD4^+$ cells (Jacobson *et al.*, 1984; Yasukawa & Zarling, 1984; Hayward *et al.*, 1986b) is increasingly recognized. Our results indicate that four of five cloned $CD4^+$ T cells with demonstrable helper activity for B cells also lysed B lymphoblastoid cells expressing the specific virus antigen. In order to determine whether this paradox was unique to the herpes virus immunity system, we tested a range of cloned T cells for cytotoxicity using a target (a CD3 antibody secreting hybridoma, UCHT1) which bypassed the requirement for antigen recognition by the T cell. CD3-making hybridomas have been used previously as targets for cytotoxicity (Perez *et al.*, 1985; Hoffman *et al.*, 1985) and we also found that their lysis was efficient even at low E:T ratios. Lysis of the UCHT1 targets was also highly specific in that a CD5-making hybridoma (UCHT2), which also binds to an antigen on T cells, was not lysed, even in the presence of cold UCHT1 targets. The latter point is relevant because it suggests that the lysis we observed was not of the 'lymphokine-activated killer' type (Phillips & Lanier, 1986), nor of bystander type described by Lanzavecchia (1986) which was characterized by low efficiency and an 8 hr rather than 4 hr time–course.

Because all the T-cell clones with definable specificity we tested lysed the UCHT1 cells, it was of interest to determine whether cytotoxicity was a property of most or all clonable T cells. The cloning efficiency of sorted $CD4^+$ or $CD8^+$ T cells cultured with irradiated autologous cells and UCHT1 cells in the presence of IL-2 ranged from 33% to 87% and, by contingency analysis, over 90% of the clones obtained were indeed cytotoxic, regardless of their CD4 or CD8 phenotype. The experimental method applied did not allow the target cell number to be adjusted according to the number of effectors per well. Consequently wells with small numbers of effectors may account for the 6–9% of proliferative but non-cytotoxic wells which were found. Support for this view comes from the positive correlation between ^{125}I-UdR uptake and ^{51}Cr release in individual wells. Exhaustion of IL-2 may account for the small number of wells in which we observed cytotoxicity but not proliferation. We interpret our data as indicating that the frequency of cytotoxicity by the CD4 and CD8 clones obtained by our culture protocol approximates to 100%, within the limits of the contingency analysis used.

Their failure of between 13% and 66% of the cells which were placed into culture after sorting to grow into clones might be due to selective or suboptimal culture conditions, or to damage to some of the sorted cells from the sorting process itself. The fact that unsorted cells had a higher cloning efficiency (70–90%) is consistent with the latter view and we were unable to obtain higher cloning efficiencies even with the PHA stimulation used by Moretta *et al.* (1983).

It might be argued that lysis of CD3 hybridoma targets is a special case because target recognition through the T-cell receptor is bypassed, and that $CD4^+$ cells might not be cytotoxic when antigen recognition is through the alpha-beta receptor heterodimer. However, this possibility seems unlikely in that the $CD4^+$ T-cell clones with 1Cl specificity lysed the 1Cl targets. Lysis of 1Cl by $CD8^+$ T-cell lines and clones has been reported previously (Boylston & May, 1986) and $CD4^+$ cells with specificity for a related idiotype, 3D6, are cytotoxic (Yssel *et al.*, 1986). Our present results extend these data in that they show that cytotoxicity by 1Cl-specific $CD4^+$ T-cell clones is a reproducible phenomenon.

Our conclusion that most of the $CD4^+$ T cells which can be cloned in the presence of IL-2 are potentially cytotoxic for an appropriate target is perhaps not surprising in view of the frequency with which cytotoxicity by CD4 clones is being reported in a range of antigen-specific systems. It is nevertheless clear that PHA does not stimulate $CD4^+$ clones to be cytotoxic, both in our own results and the larger study by Mingari *et al.* (1984).

Our results may provide some explanation for the special culture conditions (e.g. low cell density and flat-bottomed wells) which are often described as necessary for optimal *in vitro* antibody responses. Although we found that resting B cells are indeed susceptible to lysis by T cells, it is clear that B cells are not uniformly lysed by T cells from which they receive help *in vivo*. The conditions under which clones are cultured (such as rapid proliferation and excess IL-2) may contribute to their cytotoxicity *in vitro* and the unique lymphoid structures in which B cells normally develop (e.g. germinal centers and Peyer's patches) may provide an environment in which B cells would be protected.

ACKNOWLEDGMENTS

These studies were supported in part by the Imperial Cancer Research Fund and NIH grants AI 18941 and HD 13733. We are grateful to Dr J. Lamb for HA 1.7 cells and review of the manuscript and to Dr David Cappelaro for the T-cell sorting.

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