CLONAL ANALYSIS OF FUNCTIONALLY DISTINCT HUMAN CD4+ T CELL SUBSETS

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Human T lymphocytes can be divided into two subpopulations on the basis of expression of CD4 or CD8 antigens. A relationship exists between these T cell subsets and the MHC gene products recognized by their TCRs. $CD4^+$ T lymphocytes interact with target cells expressing MHC class II gene products, whereas $CD8^+$ T cells interact with target cells expressing class I MHC molecules (1). CD4 is an adhesion molecule (2) that may act as a signal transducer (3) and may form complexes with TCR in class II MHC recognition (4). Generally $CD4^+$ cells are regarded as helper cells and $CD8^+$ as cytotoxic cells. A functional distinction between $CD4^+$ and $CD8^+$ T cells is not absolute, since cloned $CD4^+$ T lymphocytes that have a cytotoxic capacity (5–9) have been described. It has been suggested that among human $CD4^+$ T cells, functionally distinct helper- and suppressor-inducer subsets exist (10–12). Recent work from several laboratories (13–16) has shown that murine $CD4^+$ T lymphocytes can be divided into at least two distinct subsets based on functional properties and lymphokine secretion. One type of helper clones (Th1) produces IL-2, IFN-γ, and TNF-β, whereas the other type (Th2) secretes IL-4 but not IL-2. Recently, new surface membrane markers have been defined that identify human T cell subpopulations (17). mAbs have been developed that identify various subsets in the $CD4^+$ T cell subset, for example CDw29 (4B4) (10) and CD27 (12), both reactive with the putative helper cells for Ig production. Other markers, i.e., Leu-8 (11) and CD45R (2H4) (18), are not expressed on the helper subset.

T cell clones obtained by direct limiting dilution from peripheral blood T lymphocytes allowed us to addresss the question whether functional heterogeneity exists at the clonal level within the human $CD4^+$ T cell subset. The cytotoxic capacity of $CD4^+$ subsets was tested with the use of mAbs against the CD3 antigen (19–22). In this way, two types of cells were obtained: $CD4^+$ T cell clones with anti-CD3-mediated cytotoxic capacity and $CD4^+$ T cell clones that were neither cytotoxic nor did they acquire anti-CD3-mediated cytotoxic activity after a culture period of 20

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wk. These functionally distinct types of CD4+ T cell clones were also shown to differ quantitatively in cytokine production and in requirements for proliferation. Our results clearly provide evidence for the existence of two fundamentally distinct types of human CD4+ T cells at the clonal level.

Materials and Methods

Cell Preparation. PBL of normal donors were prepared by Ficoll-Isopaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density-gradient centrifugation. T and non-T cells were separated by sheep E-rosette sedimentation. Residual T cells were removed from non-T cells by depletion with anti-CD3 mAbs (OKT3) plus complement.

Cloning of Lymphocytes from PBL. PBL were cloned by a direct limiting dilution. PBL were seeded in wells of Terasaki microtiter plates at an average of 0.5 cell/well in the presence of 2×10^4 irradiated (3,000 rad) autologous PBL in 20 μl of cloning medium, consisting of RPMI 1640 medium, supplemented with 10% pooled human serum and 20% of IL-2-conditioned medium, (Con A supernatant), and 0.5% PHA was added. Colonies were grown in IL-2-containing medium which was partially replaced every 3–4 d. Every 7 d, irradiated PBL were added as feeder cells together with cloning medium.

Cytotoxicity Assay. The cytotoxic activity of the T cell clones was assayed in a 4-h ^{51}Cr-release assay. As target cells we used Daudi, K562, and P815 and also Daudi and P815 in the presence of anti-CD3 mAb (CLB-T3/4.1, 1:1,000 dilution of ascites). Target cells were labeled for 1 h at 37°C with Na(^{51}Cr)O_4 (Radiochemical Centre, Amersham, United Kingdom) and were washed twice with assay medium (RPMI 1640 supplemented with 10% FCS, 1% MEM, nonessential amino acids, and antibiotics). Next, 0.1 ml assay medium containing 5,000 target cells was added to each well containing 4×10^4 effectors (T cell clone cells). The percentage of specific ^{51}Cr release was calculated as 100 × [(release by CTL − spontaneous release)/(detergent release − spontaneous release)].

Monoclonal Antibodies. OKT4 (anti-CD4) and OKT8 (anti-CD8) antibodies were purchased from Ortho Diagnostics (Oss, The Netherlands). Anti-CD28 mAb (9.3 and Kolt-2) (27) and 4B4, 2H4, and Leu-8 mAbs were obtained via the exchange of the Third International Workshop on Human Leukocyte Differentiation Antigens. The mAb R3E2, which recognizes a monomorphic determinant on MHC class II antigens (23), was provided by Dr. M. A. de Rie (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service), and mAbs specific for CD3 (CLB T3/4.1, CLB T3/4.E) (24), CD25 (CLB IL-2R/1), CD27 (CLB CD27/2) (12), and CD28 (15E8) were produced in this institute.

Indirect Immunofluorescence Assay. Cells were incubated for 30 min with a saturating amount of mAb. After two washing steps with PBS containing 0.5% (vol/vol) BSA supplemented with 0.02% (wt/vol) sodium azide (PBS-BSA), cells were incubated with FITC-conjugated goat-anti mouse antibodies for 30 min. Cells were washed three times with PBS-BSA and analyzed on a FACSCAN (Becton Dickinson & Co., Mountain View, CA).

Quantification of Cytokine Production. Factor production (IL-2, IFN-γ, IL-4, TNF) was determined after stimulation of the T cell clones (40,000 cells) with anti-CD3 mAb (CLB T3/4.E, 0.1 μg/ml) in the presence of PMA (1 ng/ml) in Iscove's modified Dulbecco's medium (IMDM),[1] supplemented with 5% FCS and antibiotics. Irradiated non-T cells were added at 8,000 cells/well. The culture supernatants were harvested at 24 and 48 h of culture and stored (−20°C) until use.

IL-2 activity was measured by the induction of proliferation in an IL-2-dependent murine T cell line (CTLL) (25).

5,000 CTLL cells were cultured in Iscove's supplemented with 5% FCS, antibiotics, and 2-ME (5×10^{-5} M) with different dilutions of T cell clone supernatants. After 16 h, the cells were labeled with [^{3}H]thymidine (2 Ci/mmol) and cultured for another 4 h before harvesting. The IL-2 concentration was related to a standard IL-2 preparation.

Quantitative measurement of IL-4 activity was kindly performed by Dr. H. Spits (Unicet

[1] *Abbreviation used in this paper:* IMDM, Iscove's modified Dulbecco's medium.

Laboratories, Dardilly, France). The ELISA assay with rabbit anti-rIL-4 IgG is described elsewhere (26).

The concentration of IFN-γ, TNF-α (cachectin), and TNF-β (lymphotoxin) after 48 h of activation of a T cell clone was determined by specific ELISAs (27). For the IFN-γ ELISA, purified rabbit anti-IFN-γ (28) was coated on U-bottomed polyvinyl chloride microtitre plates (Dynatech Laboratories, Alexandria, VA). After a washing step, samples and serial dilutions of an IFN-γ standard were added and incubated at 37°C for 1 h. The wells were washed again followed by addition of purified 4S.B3 mAb against IFN-γ (28). Next, biotinylated sheep anti-mouse Ig (Amersham International, Amersham, UK) and streptavidin-biotinylated horseradish peroxidase complex (Amersham International), respectively, were added. Finally the substrate, orthophenylenediamine, 1 mg/ml in 0.1 M citrate/phosphate buffer containing 0.006% H_2O_2, was added. The reaction was stopped after 30 min by addition of 50 μl 1 M H_2SO_4. Levels of IFN-γ present in the culture supernatants were interpolated from the IFN-γ standard calibration curve. This ELISA has a detection limit of ~1 IU/ml.

The ELISAs for TNF-α/β were carried out in a manner similar to that described for the IFN-γ ELISA except that rabbit and mouse antibodies against IFN-γ were substituted by TNF-α and -β-specific antibodies, respectively (29). For the TNF-α-specific ELISA, the mAb designated 3-101-23, giving a detection limit of ~1 U (25 pg/ml) was used. For the TNF-β-specific ELISA, the mAb L81-11 giving a detection limit of ~2 U (20 pg/ml) was used. All three ELISAs are highly specific for their respective cytokines and no crossreactivity is observed.

T Cell Proliferation Assay. Cloned T cells (4×10^4) were cultured in round-bottomed microtiter plate wells containing RPMI 1640 medium, supplemented with 10% pooled human serum and antibiotics. The cells were stimulated with 0.5% PHA (Difco Laboratories, Detroit, MI) and IL-2-containing Con A supernatant (clone culture medium), with rIL-2 (25 U/ml) and soluble anti-CD3 mAb (CLB T3/4.E, 0.1 μg/ml) in the presence or absence of PMA (1 ng/ml). Irradiated (3,000 rad) non-T cells were added at 8,000 cells/well where indicated. Cloned T cells were also stimulated with anti-CD3 mAb (CLB T3/3) immobilized on microtiter plates with or without the addition of anti-CD28 (15E8). Cultures were performed in a final volume of 200 μl of culture medium for 3 d at 37°C in a 5% CO_2 atmosphere. [^{3}H]Thymidine was added during the last 4 h of culture and the results are expressed as mean counts per minute of triplicate cultures.

T Cell-dependent Ig Synthesis. For the analysis of helper activity, graded numbers of cloned T cells (1,250-20,000 cells) were cultured in the presence of anti-CD3 mAb (CLB T3/4.E, 0.1 μg/ml) (24) and 20,000 non-T cells of a healthy donor with or without rIL-2 at 10 U/ml. Cells were cultured in round-bottomed microtiter plates (Greiner, Nürtingen, FRG) in a final volume of 170 μl Iscove's supplemented with 10% FCS (Flow Laboratories) and antibiotics. After 7 d of culture, the supernatant was harvested and Ig production was measured by an ELISA as described before (30).

Results

Analysis of Cytotoxic Capacity of CD4$^+$ T Cell Clones. Peripheral blood cells were cultured by direct limiting dilution with irradiated feeder cells in medium supplemented with IL-2-containing Con A supernatant and stimulated with PHA. Growing colonies were expanded with IL-2 and stimulated weekly with PHA. Samples of these clones were assayed for cytotoxic capacity against a panel of cell lines: Daudi, K562, P815 with or without anti-CD3 mAb, or PHA. Anti-CD3 antibodies induce cytotoxicity by bridging the CD3/TCR complex of the effector cell and the IgG-FcR of the target cell (22, 31). The cytotoxic activity of 12 CD4$^+$ T cell clones is shown in Table I. A clone was considered cytotoxic if lysis of the target cell was >20% at an E/T cell ratio of 8:1. In the absence of anti-CD3, no lytic activity against Daudi, P815, and K562 target cells was observed. Thus, these clones did not display NK cell activity and/or lymphokine-activated killer (LAK) activity. When P815 cells were used as targets, a number of CD4$^+$ clones (6/12) showed lysis in the presence of anti-

TABLE I
Cytotoxic Activity of CD4+ T Cell Clones

Clone	^{51}Cr release*			
	Daudi	K562	P815	P815 anti-CD3
		%		
103	0	3	2	6
107	0	0	0	10
110	0	1	0	1
111	0	0	0	9
116	0	3	0	2
120	0	3	5	15
108	0	4	1	48
115	0	1	1	45
105	0	1	0	40
109	0	2	0	28
113	0	2	1	37
118	0	2	0	74

* Percentage lysis during a 4-h ^{51}Cr-release assay at an E/T ratio of 8:1. Cytotoxicity was measured 7 wk after limiting dilution.

CD3 mAb. With Daudi cells as targets, 2 of 12 clones showed cytotoxicity in the presence of anti-CD3 mAb after 7 wk of culture. After 11 wk of culture, four other clones had also become cytotoxic in this system. But, as is shown in Fig. 1, these clones were already cytotoxic at week 7, when they were tested against the more susceptible target P815 plus anti-CD3 mAb. In contrast, six clones did not acquire cytotoxic activity even after a culture period of 20 wk. Thus, cytotoxic capacity appears to be a stable feature of these T cell clones.

These observations led to the hypothesis that two types of CD4+ T cell clones, with cytotoxicity (Th1) and without anti-CD3-mediated cytotoxicity (Th2), can be distinguished. It still remained to be established whether CD4+ CTL represent a true subset or are merely cells in different activation stages.

Culture Conditions Affected the Lytic Activity of CD4+ T Cell Clones. Because IFN-γ, as well as factors present in supernatants of Con A-stimulated PBL (Con A sup), has been found to play a role in the generation of cytotoxic T lymphocytes (32–34), T cell clones were cultured in the presence of rIFN-γ or high concentrations of Con A supernatant. Treatment of the T cell clones with 1,000 U rIFN-γ (TNO, Rijswijk, The Netherlands) did not induce any cytotoxic activity (Table II). Short term (4 wk) culture of all cloned T cells in high concentrations of Con A supernatant induced promiscuous lytic activity, in that both NK-sensitive and NK-resistant target cells were lysed (Table II).

Examination of Cell Surface Phenotype. To correlate the anti-CD3-mediated cytotoxic activity of CD4+ T cell clones with the expression of activation-related and subset-specific antigens, immunofluorescence studies were performed. Flow cytofluorometric analysis showed that both CD4+ Th1 and Th2 clones had a comparable high expression of MHC class II antigens and the IL-2-R (CD25) (Fig. 2). All clones showed expression of CDw29 (4B4), which has been postulated to be associated with

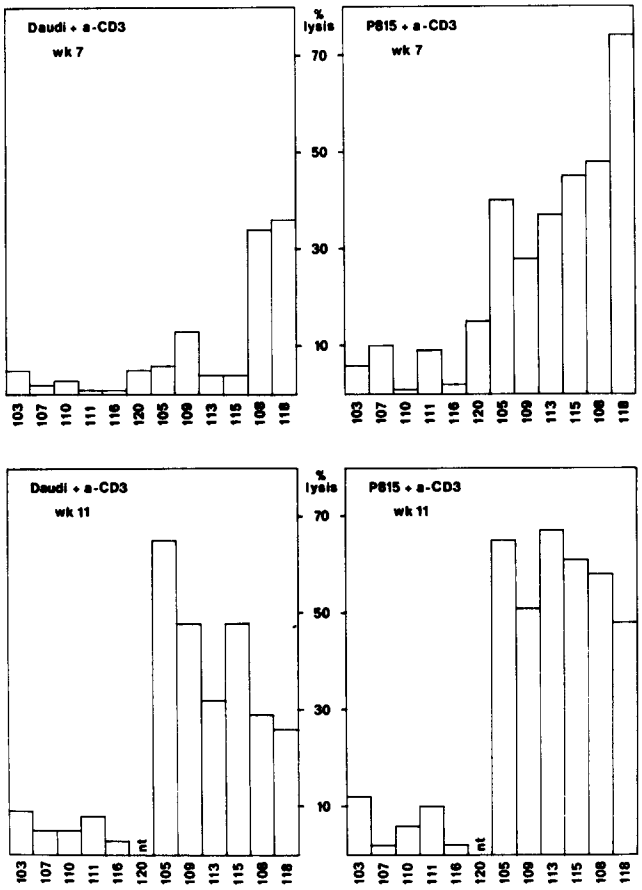

FIGURE 1. Cytotoxic activity of 12 CD4+ T cell clones. Data represent percentage lysis during a 4-h ^{51}Cr-release assay at an E/T ratio of 8:1. Early during the culture period (week 7), clones 108, 109, and 115 only show cytotoxic activity on P815 target cells in the presence of anti-CD3 mAb (CLB-T3/4.1).

TABLE II

Culture Conditions Affect the Lytic Activity of CD4+ T Cell Clones

	^{51}Cr Release								
	Daudi			K562			Daudi + anti-CD3		
Clones	Standard*	50% Con A sup.	+IFN-γ‡	Standard*	50% Con A sup.	+IFN-γ‡	Standard*	50% Con A sup.	+IFN-γ‡
	%			%			%		
103	0	42	0	1	43	0	0	63	0
116	0	48	0	0	47	1	0	70	0
108	0	13	0	0	15	0	57	51	65
115	0	69	0	8	65	10	34	72	32

The cytotoxic activity of Th2 CD4+ T cell clones (103, 116) and Th1 CD4+ T cell clones (108, 115) was measured after 3 wk of culture under different conditions.

* 20% Con A supernatant

‡ 20% Con A supernatant and 1,000 U rIFN-γ.

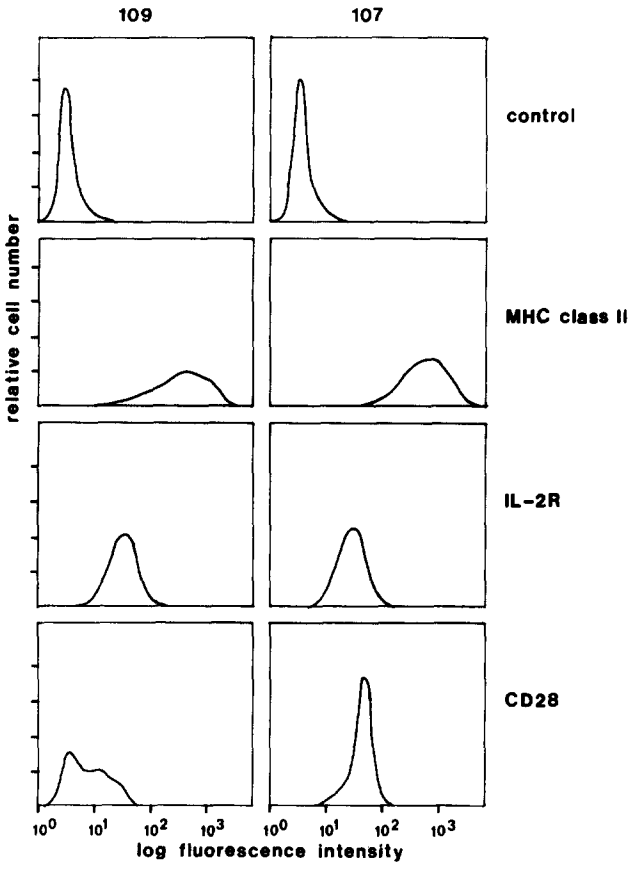

FIGURE 2. Expression of MHC class II antigen (R3E2), IL-2-R (CLB-IL-2R/1), and CD28 (Kolt-2) representative of CD4+ T-cell clones 107 (Th2) and 109 (Th1), as measured by immunofluorescence.

CD4+ helper cells. In contrast, no expression of CD45R (2H4) and Leu-8 (associated with CD4+ suppressor/inducer cells) could be demonstrated. In agreement with our previous findings (35), CD27 expression of these clones was low. On the other hand, a marked higher expression of CD28 recognized by Kolt-2, 15E8, and 9.3 mAbs on Th2 CD4+ clones as compared with Th1 CD4+ cells (Table III) was observed. Although the fluorescence profile of the low expression of CD28 on the Th1 CD4+ T cell clone appears not always homogeneous, we know from subcloning experiments that all T cell lines studied are clonal.

Lymphokine Production Profiles of CD4+ T Cell Clones. To determine whether the anti-CD3-mediated cytotoxic activity of T cell clones correlated with secretion of lymphokines, culture supernatants harvested 24 and 48 h after stimulation with anti-CD3 and PMA were assayed for lymphokines. Whereas the Th1 CD4+ clones produced substantial amounts of IL-2, IFN-γ, and TNF-α/β upon stimulation, only minimal levels of IL-2 and low levels of IFN-γ and TNF-α/β could be detected in the supernatants of the Th2 clones (Table IV). Although not all CD4+ clones did release IL-4, there was no correlation with cytotoxic activity.

Requirements for Proliferation of the CD4+ Subsets. To assess requirements for proliferation, cloned T cells were stimulated in various ways. The results presented

TABLE III
Phenotypic Analysis

Clone	Phenotype (function)	-*	CD4	CD8	4B4‡	2H4§	Leu-8	CD27	CD28
103	CD4+	2	100	13	98	4	18	5	97
107	(Th2)	1	100	2	100	4	5	21	98
110		1	100	3	100	3	11	8	100
111		1	100	1	99	2	22	21	100
116		1	100	3	96	3	3	1	100
120		2	100	2	100	4	13	6	99
105	CD4+	2	100	3	99	9	31	26	79
108	(Th1)	1	100	3	98	4	34	5	59
109		0	100	3	100	1	12	9	38
113		3	100	3	96	8	4	2	79
115		2	100	2	100	9	16	12	54
118		1	100	2	99	12	9	3	58

Surface marker analysis. Data represent percentage of positive cells.
* Background control, incubation with the FITC-labeled antibody only.
‡ CDW29.
§ CD45R.

in Table V show that no differences in proliferation of the two types of CD4+ T cell clones were found after stimulation with PHA plus Con A supernatant, rIL-2, PMA alone, and the combination of soluble or immobilized anti-CD3 and PMA. However, differences were observed after stimulation of the clones with soluble anti-CD3 mAb, i.e., the cytotoxic CD4+ T cell clones proliferated better. Anti-CD3 mAb

TABLE IV
Lymphokine Production by T Cell Clones

Clone	Phenotype (function)	IL-2*	IL-4*	IFN-γ‡	TNF-α‡	TNF-β‡
		U/ml	*pg/ml*	*IU/ml*	*U/ml*	*U/ml*
103	CD4+ (Th2)	3.6	240	22	34	15
107		<1	–§	25	22	8
110		<1	–	39	24	24
111		<1	–	25	17	4.5
116		<1	170	<4	18	<1
120		<1	120	15.5	34	<1
105	CD4+ (Th1)	38.4	125	100	160	90
108		4.8	–	55	112	73
109		4.6	–	85	88	20
113		2.4	275	78	66	30
115		76.8	120	35	208	120
–‖		<1	–	<2	4	<1

To measure lymphokine release, supernatants were collected after 1-d* or 2-d‡ cultures of 4×10^4 T cells with 8,000 irradiated non-T cells (3,000 rad) incubated with anti-CD3 (CLB-T3/4.E: 0.1 μg/ml) and PMA (1 ng/ml).
§ –, below detection level.
‖ Feeder cells incubated for the indicated time period without cloned T cells.

TABLE V

Differences in Requirements for Proliferation between the CD4 Subsets

Clones	Phenotype (function)	PHA, Con A sup*	rIL-2	Control*	α-CD3*	α-CD3 PMA*	PMA*	Immobilized anti-CD3 Alone	Immobilized anti-CD3 Plus anti-CD28
103	CD4+	2,458	ND	47	191	3,575	50	6,500	12,600
107	(Th2)	5,199	13,700	185	210	5,578	332	6,200	8,400
110		2,770	1,400	51	113	4,680	51	1,300	2,900
111		6,702	3,100	30	169	3,245	39	3,200	10,100
116		3,255	3,800	41	143	4,398	32	ND	ND
120		5,073	8,800	25	179	5,098	48	6,300	12,600
105	CD4+	5,619	16,200	47	3,562	3,128	100	9,000	9,100
108	(Th1)	6,569	10,700	53	1,746	7,028	155	7,400	7,500
109		3,787	14,160	32	1,015	10,361	95	14,000	12,700
113		3,529	7,100	38	338	12,773	65	7,900	9,400
115		2,943	6,700	37	1,541	4,212	59	6,000	6,400
T		6,796	400	54	4,869	8,845	609	9,500	8,100

The values present the mean of triplicate cpm values.

* Irradiated normal non-T cells, as monocyte sources were added.

immobilized on microtiter plates induced proliferation of the T cell clones. Proliferation in relation to the density of immobilized anti-CD3 mAb was identical for both subsets (data not shown). Interestingly, differences were observed when the immobilized anti-CD3 system in combination with anti-CD28 mAb was used. The proliferation of the CD4+ T cell clones lacking anti-CD3-mediated cytotoxicity and with high CD28 expression (Th2), was enhanced twofold by addition of anti-CD28 mAb in the immobilized anti-CD3 system (Table V). On the other hand, addition of anti-CD28 mAb had no effect on the proliferation induced by immobilized anti-CD3 of cytotoxic CD4+ cells (Th1), which have a low expression of CD28.

Helper Activity of T Cell Clones for Polyclonal B Cell Differentiation. T helper activity for B cell differentiation was tested in a polyclonal system where T helper activity is induced by anti-CD3 mAb as described before (36, 37). This induction of helper activity is only partially dependent on the release of IL-2, which in turn induces helper factor release. IL-2 alone in this system does not directly induce B cell differentiation (36). The responsive B cell as in the PWM-driven polyclonal system is a preactivated B cell (38). To investigate the possibility that lack of helper activity was due to low IL-2 production from T cells induced by anti-CD3 mAb, exogeneous IL-2 was added. Differences in helper activity in the presence of IL-2 are most likely due to differences in release of T cell helper factors for B cell differentiation (12, 39). Both types of CD4+ T cell clones demonstrated helper activity for B cell IgM and IgG production upon anti-CD3 stimulation in the absence and presence of exogenous IL-2 (Fig. 3). Because of the low number of T cell clones tested, conclusive statistical analysis of the differences in helper activity for IgM and IgG synthesis between the two CD4+ subsets upon anti-CD3 stimulation in the presence and absence of IL-2 could not be performed.

Discussion

In this report, we describe two types of functionally distinct cloned human CD4+ T cells. CD4+ T cell clones with cytotoxic capacity (Th1) were good producers of

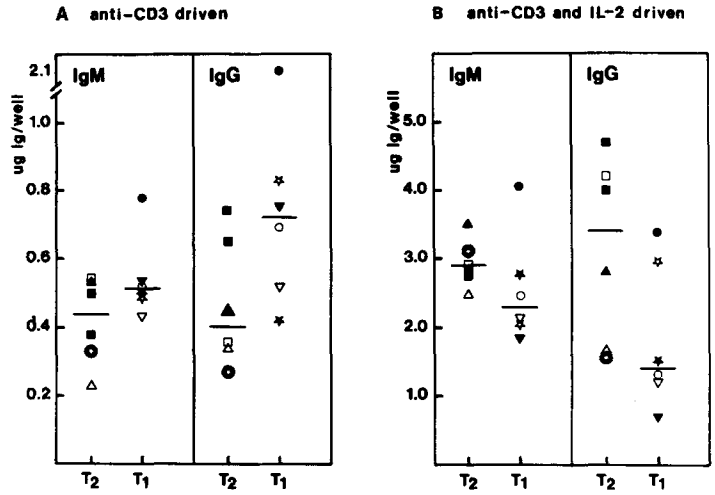

FIGURE 3. Helper activity of T cell clones on IgM and IgG production. 2,000 cloned T cells of $CD4^+$ noncytotoxic $CD4^+$ T cell clones (T2): 103 (▲), 107 (△), 110 (■), 111 (□), 116 (XXX) and 120 (XXX) and $CD4^+$ cytotoxic T cell clones (T1): 105 (▼), 108 (▽), 109 (XXX), 113 (XXX), 115 (●), and 118 (○) were stimulated with anti-CD3 mAbs in the absence (*A*) or presence (*B*) of 10 U rIL-2. The background Ig production for non-T (20,000) + anti-CD3 mAb (*A*) is <0.15 μg IgM/well and <0.09 μg IgG/well and the background level for stimulation with anti-CD3 mAbs and IL-2 (*B*) are <0.65 μg IgM/well and <0.5 μg IgG/well.

IL-2, IFN-γ, and TNF-α/β, and $CD4^+$ T cell clones without anti-CD3-mediated cytotoxicity (Th2) produced little IL-2 and only low levels of IFN-γ and TNF-α/β. The $CD4^+$ T cell clones with high CD28 expression proliferated better in response to the combination of anti-CD3 and anti-CD28 mAb. The two distinct types of $CD4^+$ T cell clones provided both good helper activity for polyclonal Ig synthesis.

The cytotoxic capacity was a stable feature of the T cell clones. During 20 wk of culture $CD4^+$ cytotoxic clones consistently lysed target cells in the presence of anti-CD3 mAb, but the noncytotoxic $CD4^+$ clones did not acquire anti-CD3-mediated cytotoxic activity in that period of time. It has been described that long term culture in the presence of IL-2 induces nonspecific cytotoxicity (40). In contrast, the anti-CD3-mediated cytotoxicity observed in this study was not the result from nonspecific cytotoxicity against NK-sensitive targets (K562) or LAK-sensitive targets (Daudi), since these clones lacked cytotoxic activity in the absence of anti-CD3.

Short term (4 wk) culture of the cloned T cells in high concentrations of Con A supernatant induced promiscuous lytic activity, in that both NK-sensitive and NK-resistent target cells were lysed. These induced cytotoxic activities are most likely mediated through receptor systems distinct from the TCR molecules (41).

Cytotoxic $CD4^+$ T cell clones obtained from polyclonally stimulated PBL have been described by Moretta et al. (42) and Weber et al. (43). In these studies, a much smaller fraction of $CD4^+$ cells/clones mediated lytic activity in lectin-dependent systems. The discrepancy may be caused by the fact that lectin-dependent cytotoxicity is not as strong as the anti-CD3-mediated cytotoxicity (data not shown). On the other hand, it has been reported that all $CD4^+$ T cell clones can acquire specific cytotoxic activity in culture (6, 8). Possibly different culture protocols, for instance

a preceding bulk culture, lead to different selection. Most data on cytotoxic CD4+ T cells concern cloned cells in long term cultures (5–9). In short term bulk cultures, virus-specific cytotoxic CD4+ T cells induced by cytomegalovirus (44) and measles virus (45) have been described, whereas for influenza virus we have shown (46) that CD4+ cytotoxic cells do not play a role in short term bulk cultures. There are observations that suggest that culture conditions may affect the differentiation of proliferative T cell clones into cytotoxic T cell clones (32–34, 47). The exact signals involved in the induction of these functional changes remain to be elucidated. Our findings demonstrate that CD4+ T cells lacking anti-CD3-mediated cytotoxic capacity can be isolated and can be stably maintained in culture by polyclonal stimulation.

Although a correlation between T cell activation and cytotoxic activity has been supposed (48), there was no evidence that the T cell clones, without anti-CD3-mediated cytotoxic activity, were in a permanently less activated state, as indicated by equal levels of IL-2-R and MHC class II antigen on both types of clones.

Phenotypic analysis of the two distinct types of CD4+ T cell clones revealed no differences with regard to expression of CDw29 (4B4) and CD45R (2H4) determinants. Whereas Morimoto et al. (10) described lymphocytes within the peripheral blood CD4+ that were functionally different subpopulations recognized by these mAbs, at the clonal level these mAbs did not make any functional distinction in CD4+ subsets. However, the two types of CD4+ T cell clones can be distinguished phenotypically with the anti-CD28 mAb. CD28 has been described as a cell surface antigen expressed on ~80% of mature T lymphocytes, including both helper cells and cytotoxic cells (49). Interestingly, for CD4+ T cell clones that lack anti-CD3-mediated cytotoxic capacity and have high CD28 expression, the proliferation, induced with immobilized anti-CD3 mAb, was increased by addition of anti-CD28 mAb. On the other hand, for CD4+ cytotoxic clones with low CD28 expression, addition of anti-CD28 mAbs had no effect on anti-CD3-induced proliferation. These findings are in agreement with the data in the literature that suggest a function for CD28 in T cell activation (50–52). Whereas CD28 is involved in T cell proliferation, anti-CD28 mAb had no effect on the cytotoxic activity of the clones (data not shown).

A correlation between cytotoxic activity and lymphokine release by T cells has been suggested (53), whereas others did not find this correlation (27, 54). We found a good correlation between CTL activity and the amount of IFN-γ, TNF-α/β production for CD4+ T cell clones. The coexpression of cytolytic and helper functions has been observed before in cloned peripheral blood T lymphocytes (55). The CD4+ clones with cytotoxic activity described by us were also good IL-2 producers and provided helper activity for Ig synthesis. We did not find any correlation of lymphokine production with proliferation in response to anti-CD3 mAb and PMA. Although the noncytotoxic CD4+ T cells produced minimal amounts of IL-2, they were able to provide help for Ig secretion by B cells upon anti-CD3 stimulation in the absence and presence of exogenous IL-2. These results could imply two pathways for help for B cell differentiation: IL-2 independent (36, 39) and IL-2 dependent (37, 56).

Recent work from several laboratories (13–16) has shown that murine CD4+ T lymphocytes can be divided into at least two distinct subsets based on function and secretion of lymphokines. The Th1 cells provide helper activity for polyclonal B cell differentiation, have cytotoxic capacity and release IL-2, IFN-γ, and TNF-β, whereas

Th2, on the other hand, give antigen-specific helper activity for B cell Ig synthesis and release IL-4 (13, 15). The helper activity for B cell differentiation provided by the clones was measured in this study on polyclonal B cell responses. It has been observed in the mouse that IL-4 is required for the induction of IgE antibody release (57). Induction of IgE synthesis in normal B cells by the clones used in the present study was not observed (data not shown). Perhaps this is due to inhibition of IgE synthesis by IFN-γ. The heterogeneity within the human $CD4^+$ subset is largely in agreement with the murine system. In the human system, like in the mouse, $CD4^+$ T cells with cytotoxic capacity do release more IL-2, IFN-γ, and TNF-β. A point of disagreement is the production of IL-4 by both types of human $CD4^+$ clones.

The role of these two types of $CD4^+$ cells in the immune response is not yet clear. Despite some differences, it can be hypothesized, in analogy to the murine system, that the main function of the noncytotoxic T cells is to provide help for B cell differentiation, whereas the cytotoxic T cells are the most important effector cells in inflammatory reactions and DTH responses.

Summary

A large number of $CD4^+$ T cell clones, obtained from peripheral blood T lymphocytes by direct limiting dilution, allowed us to address the question whether functional heterogeneity exists within the human $CD4^+$ T cell subset. Cytotoxic capacity of cloned T cells was analyzed with the use of anti-CD3 antibodies and target cells bearing FcR for murine IgG. 6 of 12 $CD4^+$ clones obtained were able to lyse Daudi or P815 cells in the presence of anti-CD3 antibodies. The remaining six $CD4^+$ T cell clones tested did not display anti-CD3-mediated cytotoxic activity and did not acquire this cytotoxic capacity during a culture period of 20 wk. In the absence of anti-CD3 mAb, no lytic activity against Daudi, P815, and K562 target cells was observed under normal culture conditions. Phenotypic analysis of these two distinct types of $CD4^+$ T cells did not reveal differences with regard to reactivity with CDw29 (4B4) and CD45R (2H4) mAbs that have been described to recognize antigens associated with helper suppressor/inducer (respectively) $CD4^+$ cells. The $CD4^+$ clones without anti-CD3-mediated cytotoxic activities (Th2) consistently showed a high expression level of CD28 antigens, whereas the cytotoxic clones (Th1) expressed low amounts of CD28. Th1 $CD4^+$ clones did produce IL-2, IFN-γ, and TNF-α/β, whereas the Th2 T cell clones produced minimal amounts of IL-2 and only low levels of INF-γ and TNF-α/β in response to anti-CD3 mAbs and PMA. Although not all $CD4^+$ clones did release IL-4, there was no correlation with cytotoxic activity. Moreover, as compared with the Th1 $CD4^+$ clones, Th2 $CD4^+$ T cell clones proliferated moderately in response to immobilized anti-CD3 mAbs. However, proliferation reached the level of the cytotoxic clones when anti-CD28 mABs were present during culture. Both $CD4^+$ subsets provided help for B cell differentiation upon stimulation with anti-CD3 mAbs. Our data suggest that the human $CD4^+$ subset, in analogy to the murine system, comprises two functionally distinct T cell subpopulations, both of which are able to exert helper activity for polyclonal B cell differentiation, but which differ in cytotoxic capacity, lymphokine production, and requirements for proliferation. A function for these two types of T cells in the immune response is discussed.

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References

1. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. USA.* 79:4395.
2. Shaw, S., G. Goldstein, T. A. Springer, and W. E. Biddison. 1985. Susceptibility of cytotoxic T lymphocyte (CTL) clones to inhibition by anti-T3 and anti-T4 (but not anti-LFA-1) monoclonal antibodies varies with the "avidity" of CTL-target interaction. *J. Immunol.* 134:3019.
3. Bank, I., and L. Chess. 1985. Perturbation of the T4 molecule transmits a negative signal to T cells. *J. Exp. Med.* 162:1294.
4. Saizawa, K., J. Rojo, and C. A. Janeway, Jr. 1987. Evidence for a physical association of CD4 and the CD3:α:β T-cell receptor. *Nature (Lond.).* 328:260.
5. Moretta, L., M. C. Mingari, P. R. Sekaly, A. Moretta, B. Chapuis, and J. -C. Cerottini. 1981. Surface markers of cloned human T cells with various cytolytic activities. *J. Exp. Med.* 154:569.
6. Yasukawa, M., and J. M. Zarling. 1984. Human cytotoxic T-cell clones directed against herpes simplex virus-infected cells. I. Lysis restricted by HLA class-II MB and DR antigens. *J. Immunol.* 133:422.
7. Jacobson, S., J. R. Richert, W. E. Biddison, A. Satinsky, R. J. Hartzman, and H. F. McFarland. 1984. Measles virus-specific T4+ human cytotoxic T-cell clones are restricted by class-II HLA antigens. *J. Immunol.* 133:754.
8. Fleischer, B. 1984. Acquisitions of specific cytotoxic activity by human T4+ T lymphocytes in culture. *Nature (Lond.).* 308:365.
9. Fleischer, B., and H. Wagner. 1986. Significance of T4 or T8 phenotype of human cytotoxic T-lymphocyte clones. *Curr. Top. Microbiol. Immunol.* 126:101.
10. Morimoto, C., N. L. Letvin, A. W. Boyd, M. Hagan, H. M. Brown, M. M. Kornacki, and S. F. Schlossman. 1985. The isolation and characterization of the human helper inducer T-cell subset. *J. Immunol.* 134:3762.
11. Kansas, G. S., G. S. Wood, D. M. Fishwild, and E. G. Engleman. 1985. Functional characterization of human T-lymphocyte subsets distinguished by monoclonal anti-leu-8. *J. Immunol.* 134:2995.
12. Van Lier, R. A. W., M. Oudkerk Pool, P. Kabel, S. Mous, F. Terpstra, M. A. de Rie, C. J. M. Melief, and F. Miedema. 1988. Anti-CD27 monoclonal antibodies identify two functionally distinct subpopulations within the CD4+ T-cell subset. *Eur. J. Immunol.* 18:811.
13. Tite, J. P., M. B. Powell, and N. H. Ruddle. 1985. Protein-antigen-specific Ia-restricted cytolytic T cells: analysis of frequency, target-cell susceptibility and mechanisms of cytolysis. *J. Immunol.* 135:25.
14. Kim, J., A. Woods, E. Becker-Dunn, and K. Bottomly. 1985. Distinct functional phenotypes of cloned Ia-restricted helper T cells. *J. Exp. Med.* 162:188.
15. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T-cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
16. Kurt-Jones, E. A., S. Hamberg, J. Ohara, W. E. Paul, and A. K. Abbas. 1987. Heteroge-

neity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J. Exp. Med.* 166:1774.
17. Beverly, P. C. L. 1986/1987. Human T-cell subsets. *Immunol. Lett.* 14:263.
18. Rudd, C. E., C. Morimoto, L. L. Wong, and S. F. Schlossman. 1987. The subdivision of the T4 (CD4) subset on the basis of the differential expression of L-C/T200 antigens. *J. Exp. Med.* 166:1758.
19. Spits, H., H. Yssel, J. Leeuwenberg, and J. E. de Vries. 1985. Antigen-specific cytotoxic T cell and antigen-specific proliferating T-cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. *Eur. J. Immunol.* 15:88.
20. Mentzer, S. J., J. A. Barbosa, and S. J. Burakoff. 1985. T3 monoclonal antibody activation of nonspecific cytolysis: a mechanism of CTL inhibition. *J. Immunol.* 135:34.
21. Schrezenmeier, H., R. Kurrle, H. Wagner, and B. Fleischer. 1985. Activation of human T lymphocytes. III. Triggering of bystander cytotoxicity in cytotoxic T-cell clones by antibodies against the T3 antigen or by a calcium ionophore. *Eur. J. Immunol.* 15:1019.
22. Van Seventer, G. A., K. C. Kuijpers, R. A. W. van Lier, E. R. de Groot, L. A. Aarden, and C. J. M. Melief. 1987. Mechanism of inhibition and induction of cytolytic activity in cytotoxic T lymphocytes by CD3 monoclonal antibodies. *J. Immunol.* 139:2545.
23. De Rie, M. A., P. Kabel, R. W. Sauerwein, R. A. W. van Lier, A. E. G. Kr. von dem Borne, C. J. M. Melief, and F. Miedema. 1987. Anti-HLA-class II monoclonal antibodies inhibit polyclonal B cell differentiation in vitro at the accessory cell level. *Eur. J. Immunol.* 17:881.
24. Van Lier, R. A. W., J. H. A. Boot, E. R. de Groot, and L. A. Aarden. 1987. Induction of T cell proliferation with anti-CD3 switch variant monoclonal antibodies: effects of heavy chain isotype in monocyte-dependent systems. *Eur. J. Immunol.* 17:1599.
25. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
26. Paliard, X., R. De Waal Malefijt, H. Yssel, D. Blanchard, I. Chrétien, J. S. Abrams, J. E. De Vries, and H. Spits. 1988. Simultaneous production of IL-2 IL-4 and IFNγ by activated human $CD4^+$ and $CD8^+$ T lymphocyte clones. *J. Immunol.* 141:849.
27. Christmas, S. E., A. Meager, and M. Moore. 1987. Production of interferon and tumor necrosis factor by cloned human natural cytotoxic lymphocytes and T cells. *Clin. Exp. Immunol.* 69:441.
28. Leist, T., R. Titmas, S. Parti, and A. Meager. 1985. Antibodies to synthetic polypeptides corresponding to hydrophilic regions of human interferon gamma. *Mol. Immunol.* 22:929.
29. Meager, A., S. Parti, H. Leung, J. Woolley, E. Peil, S. Sidhu, and T. Roberts. 1987. A two-site sandwich assay of human lymphotoxin with monoclonal antibodies and its applications. *J. Immunol. Methods.* 104:31.
30. Rümke, H. C., F. Miedema, R. J. M. ten Berge, F. Terpstra, H. J. van der Reijden, R. J. van de Griend, H. G. de Bruin, A. E. G. Kr. von dem Borne, J. W. Smit, W. P. Zeijlemaker, and C. J. M. Melief. 1982. Functional properties of T cells in patients with chronic Tγ lymphocytosis and chronic T-cell neoplasia. *J. Immunol.* 129:419.
31. Griend, R. J. van de, R. L. H. Bolhuis, G. Stoter, and R. C. Roozemond. 1987. Regulation of cytolytic activity in $CD3^-$ and $CD3^+$ killer-cell clones by monoclonal antibodies (anti-CD16, anti-CD2, anti-CD3) depends on subclass specificity of target-cell IgG-FcR. *J. Immunol.* 138:3137.
32. Raulet, D. H., and M. J. Bevan. 1982. A differentiation factor required for the expression of cytotoxic T-cell function. *Nature (Lond.).* 296:754.
33. Chen, L. -K., A. Bensussan, G. F. Burns, B. Tourvieille, A. Soulié, and M. Sasportes. 1986. Allospecific proliferative human T-cell clones acquire the cytotoxic effector function after three months in culture, in IL-2-conditioned medium. *Hum. Immunol.* 17:30.
34. Chen, L. -K., D. Mathieu-Mahul, M. Sasportes, L. Degos, and A. Bensussan. 1986.

What is a T-cell clone? Effect of rIFN on T-cell-clone function and T-cell-receptor gene rearrangement. *Hum. Immunol.* 17:214.

35. Van Lier, R. A. W., J. Borst, T. M. Vroom, H. Klein, P. van Mourik, W. P. Zeijlemaker, and C. J. M. Melief. 1987. Tissue distribution and biochemical and functional properties of Tp55 (CD27), a novel T cell differentiation antigen. *J. Immunol.* 139:1589.
36. Sauerwein, R. W., W. G. J. van der Meer, J. W. van Oostveen, F. Miedema, and L. A. Aarden. 1988. Anti-CD3 antibodies induce T-helper function for human B-cell differentiation in vitro by an interleukin-2-independent pathway. *Eur. J. Immunol.* 18:133.
37. Stohl, W., D. N. Posnett, and N. Chiorazzi. 1987. Induction of T cell-dependent B cell differentiation by anti-CD3 monoclonal antibodies. *J. Immunol.* 138:1667.
38. Kuritani, T., and M. D. Cooper. 1982. Human B cell differentiation II Pokeweed mitogen-responsive B cells belong to a surface immunoglobulin D-negative subpopulation. *J. Exp. Med.* 155:1561.
39. Lamb, J. R., E. D. Zanders, M. Feldmann, P. Lake, D. D. Eckels, J. N. Woody, and P. C. L. Beverly. 1983. The dissociation of interleukin-2 production and antigen-specific helper activity by clonal analysis. *Immunology.* 50:397.
40. Brooks, C. G., D. L. Urdal, and C. S. Henney. 1983. Lymphokine-driven "differentiation" of cytotoxic T-cell clones into cells with NK-like specificity: correlations with the display of membrane macromolecules. *Immunol. Rev.* 72:43.
41. Brooks, C. G., and M. Holscher. 1987. Cell-surface molecules involved in NK recognition by cloned cytotoxic T lymphocytes. *J. Immunol.* 138:1331.
42. Moretta, A., G. Pantaleo, L. Moretta, M. C. Mingari, and J. -C. Cerottini. 1983. Quantitative assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T-cell populations. *J. Exp. Med.* 158:571.
43. Weber, W. E. J., W. A. Buurman, M. M. P. P. Vandermeeren, and J. C. M. Raus. 1985. Activation through CD3 molecule leads to clonal expansion of all human peripheral blood T lymphocytes: functional analysis of clonally expanded cells. *J. Immunol.* 135:2337.
44. Lindsley, M. D., D. J. Torpey III, and C. R. Rinaldo. 1986. HLA-DR-restricted cytotoxicity of cytomegalovirus-infected monocytes mediated by leu-3-positive T cells. *J. Immunol.* 136:3045.
45. Jacobson, S., J. Rose, M.-L. Flerlage, E. E. Mingoli, D. E. McFarlin, and H. F. McFarland. 1987. Measles virus specific human cytotoxic T cells generated in bulk cultures. Analysis of measles virus antigenic specificity. *In* Biology of Negative Strand Viruses. B. Mahy and D. Kolakofsky, editors. Elsevier Science Publishers B. V., Amsterdam. 283–289.
46. Rotteveel, F. T. M., E. Braakman, B. Robbe, and C. J. Lucas. 1988. Recognition of influenza virus-infected B-cell lines by human influenza virus-specific CTL. *Cell. Immunol.* 111:473.
47. Takai, Y., S. H. Herrmann, J. L. Greenstein, G. L. Spitalny, and S. J. Burakoff. 1986. Requirement for three distinct lymphokines for the induction of cytotoxic T lymphocytes from thymocytes. *J. Immunol.* 137:3494.
48. Jung, G., C. J. Honsik, R. A. Reisfeld, and H. J. Müller-Eberhard. 1986. Activation of human peripheral blood mononuclear cells by anti-T3: killing of tumor target cells coated with anti-target-anti-T3 conjugates. *Proc. Natl. Acad. Sci. USA.* 83:4479.
49. Hansen, J. A., P. J. Martin, and R. C. Nowinski. 1980. Monoclonal antibodies identifying a novel T-cell antigen and Ia antigens of human lymphocytes. *Immunogenetics.* 10:247.
50. Van Lier, R. A. W., M. Brouwer, and L. A. Aarden. 1988. Signals involved in T cell activation. T cell proliferation induced through the synergistic action of anti-CD28 and anti-CD2 monoclonal antibodies. *Eur. J. Immunol.* 18:167.
51. Hara, T., S. Man Fu, and J. A. Hansen. 1985. Human T cell activation. II. A new activation pathway used by a major T cell population via a disulfide-bonded dimer of a 44 kilodalton polypeptide (9.3 antigen). *J. Exp. Med.* 161:1513.

52. Moretta, A., G. Pantaleo, M. Lopez-Botet, and L. Moretta. 1985. Involvement of T44 molecules in an antigen-independent pathway of T cell activation. Analysis of the correlations to the T cell antigen-receptor complex. *J. Exp. Med.* 162:823.
53. Green, L. M., J. L. Reade, C. F. Ware, P. E. Devlin, C. -M. Liang, and J. J. Devlin. 1986. Cytotoxic lymphokines produced by cloned human cytotoxic T lymphocytes. II. A novel CTL-produced cytotoxin that is antigenically distinct from tumor necrosis factor and α-lymphotoxin. *J. Immunol.* 137:3488.
54. Klein, J. R., M. S. Pasternack, and M. J. Bevan. 1985. Activation requirements for antigen- and mitogen-induced interferon-γ release from cytotoxic T lymphocytes. *J. Immunol.* 134:2456.
55. Mingari, M. C., A. Moretta, E. Maggi, G. Pantaleo, F. Gerosa, S. Romagnani, and L. Moretta. 1984. Frequent coexpression of cytolytic activity and lymphokine production among human T lymphocytes. Production of B-cell growth factor and interleukin 2 by $T8^+$ and $T4^+$ cytolytic clones. *Eur. J. Immunol.* 14:1066.
56. Miedema, F., J. W. van Oostveen, F. G. Terpstra, A. W. L. van den Wall Bake, R. Willemze, E. A. J. Rauws, R. Bieger, M. B. van 't Veer, D. Catovsky, and C. J. M. Melief. 1985. Analysis of helper activity on pokeweed mitogen- and interleukin 2-driven immunoglobulin synthesis by neoplastic $T4^+$ cells. *J. Clin. Invest.* 76:2139.
57. Coffman, R. L., and J. Carty. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-γ. *J. Immunol.* 136:949.