POSSIBLE INVOLVEMENT OF THE T4 MOLECULE IN T CELL RECOGNITION OF CLASS II HLA ANTIGENS

Evidence from Studies of CTL-Target Cell Binding

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Evidence for the functional involvement of T cell differentiation antigens inT cell recognition and/or activation has been provided by various studies in which antibodies to certain of these antigens have been shown to inhibit antigen-specific cytotoxicity (1–16), proliferation (17–19), and release of lymphokines (16, 20, 21). This phenomenon has been most extensively documented by studies that have utilized antibodies directed against the T8 molecule (22) (Lyt-2 in mouse), the T4 molecule (22) (L3T4 in mouse [23]), and the T3 molecule (22). To date, the available data suggest that the T3, T4, and T8 molecules are involved in T cell recognition or activation and are not involved in the lytic process. Models have been proposed in which the T4 and T8 molecules bind to nonpolymorphic structures on class II and class I MHC antigens (10, 16, 24), respectively, and that the T3 molecule is intimately associated with the T cell antigen-specific receptor (24).

Studies in mice have shown variation in the susceptibility of different T cell populations (clones and lines) to the inhibitory effect of anti–Lyt-2 antibodies (6, 25). Our previous studies have documented variability in the susceptibility of SB-specific cytolytic T lymphocyte (CTL)[1] populations to inhibition by anti-T4 antibodies (10). These observations have given rise to the hypothesis that the T4 molecule may play a role in facilitating interactions of low affinity clones but may be irrelevant to the recognition process of high affinity clones (6, 10, 25). The present study explores the relationship between the susceptibility of CTL clones to inhibition by anti–T cell antibodies and the tightness with which they bind targets as estimated by a previously published assay of CTL–target cell conjugate dissociation. In addition, this dissociation assay partitions the lytic process into binding vs. lethal hit phases and provides a more direct demonstration of the role of the T4 molecule in target cell binding.

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocyte; EGTA, ethyleneglycol-bis-(B-amino-ethyl ether *N,N'*-tetra-acetic acid); FMF, flow microfluorometry; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; PBL, peripheral blood lymphocytes; PHA-sup, supernatant from PHA-stimulated PBL; TCGF, T cell growth factor.

Materials and Methods

Human Blood Reagents. Peripheral blood mononuclear leukocytes (PBL) and plasma were obtained by batch leukapheresis of normal adult volunteers (26). PBL were separated by flotation on Ficoll-Hypaque and were cryopreserved as described (26). Plasma from 5–10 such male donors was pooled, frozen in aliquots at −20°C, and used as the normal human plasma pool. HLA serotyping of cells was kindly performed by Dr. R. J. Duquesnoy, Blood Center of Southeastern Wisconsin, Milwaukee, WI. SB typing was performed by primed lymphocyte typing as previously described (27–29). The HLA phenotypes of the donors used in this study are given in Table I.

Immunochemical Reagents. Purified monoclonal antibodies were prepared from ascites as described (22). The monoclonal antibodies used in these studies are OKT4 (IgG2b) (22), OKT4A (IgG2a) (30), OKT4B (IgM) (30), OKT8A (IgG2) (31), and OKT3 (IgG2a) (22). An additional antibody to the T4 molecule, OKT4F (IgG1), was produced and characterized as previously described for OKT4, 4A, and 4B (22, 30). Protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ)–purified anti-Tac (IgG2a) (32, 33) was the generous gift of Dr. T. Waldmann (National Cancer Institute, Bethesda, MD). Anti-Tac binds to the cell surface receptor for T cell growth factor (TCGF) (32, 33).

Generation and Assay of Cloned SB-Specific CTL. Bulk CTL effector cells were generated in primary and secondary mixed lymphocyte culture (MLC) as previously described (27–29). Responder and stimulator cells were matched for HLA-A, -B, -C, -D, -DR, MB, and MT but mismatched for SB; responder-stimulator combinations were identical to those used to generate the standard primed lymphocyte typing reagents that define the SB specificities (27–29). Secondary MLC cells were stimulated with specific stimulators for 2–3 d then cloned at 3 and 0.3 cells/well in flat-bottom 96-well plates (Costar #3596; Costar, Data Packaging, Cambridge, MA) in complete medium consisting of RPMI 1640 with glutamine (Grand Island Biological Co., Grand Island, NY and Biofluids, Inc., Rockville, MD) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% normal human plasma. Wells were supplemented with 40% supernatant from PHA-stimulated PBL (PHA-sup, see below) and 10^5 6000R PBL feeder cells that expressed the relevant SB antigen. 10–14 d later wells with visible cell growth were assayed for cytolytic activity. Cytolytic clones were expanded repeatedly as follows: ~10^5 cells were transferred to a 2-cm^2 well (Linbro #76-033-05; Linbro Chemical Co., Hamden, CT) containing 1 × 10^6 6000R stimulator PBL in 1.5 ml complete medium. 24 h later each well received 0.4 ml PHA-sup. 5–6 d later rapidly growing clones were reexpanded once more or cryopreserved (26) before further expansion. All T cell clones used in the present study were derived from initial cloning at 0.3 cells/well or were recloned at that concentration.

The standard ^{51}Cr release cytotoxicity assay was performed as previously described (26). Effector cells were assayed 5 d after stimulation with PBL stimulators and 18–24 h

TABLE I

HLA Phenotypes of Donors

Donor	HLA-A	-B	-C	-DR	-SB	MB	MT
B17	1,2	7,8	7	2,4	2,4	1,3	1,3
C5	1,3	8	—	3,5	2,4	2,3	2
F2	1,2	7,8	—	2,4	4,5	1,3	1,3
FB6	25,31	15,40	3	4,4	4	3	3
FB11	25,31	15,40	3	4,4	1	3	3
H9	1,2	7,8	7	2,3	1,2	1,2	1,2
K4	1,2	7,8	7	1,3	1	1,2	1,2
M14	1,2	7,8	7	2,3	3,4	1,2	1,2
M16	1,3	8,14	—	2,3	1,2	1,2	1,2
PM1	1,2	7,8	7	2,3	2,3	1,2	1,2
S11	1,2	7,8	7	2,3	2,5	1,2	1,2
W7	1,2	7,8	7	2,3	1,4	1,2	1,2

after boosting with fresh stimulators. Target cells were lymphoblastoid B cell lines transformed with Epstein-Barr virus (27). In antibody-blocking assays, effector cells were preincubated with antibodies 15–20 min at 37°C before addition of ^{51}Cr-labeled target cells; incubation times for these assays ranged from 1.5–4 h and were chosen so as to achieve unblocked lyses in an optimum range (20–50% specific lysis).

Generation of PHA-sup. Growth media for T cell clones was generated by PHA-stimulation of PBL from selected donors. 80 × 10^6 PBL, 12 × 10^6 lymphoblastoid B cells from donor H9 (H9B), 0.8 ml PHA M form (Gibco), and 0.8 ml normal human plasma plus RPMI 1640 with penicillin and streptomycin in a final volume of 80 ml were cultured in an upright plastic flask (Falcon #3023; Falcon Labware, Oxnard, CA) for 48 h. The supernatant was harvested, centrifuged to remove cells, filtered through a 0.45-μm filter (Nalge Co., Rochester, NY), and frozen in aliquots at −70°C. The growth-promoting activity of the supernatant was assayed by its ability to promote growth of cloned CTL.

Assay for Dissociation of Functional CTL–Target Cell Conjugates. This assay was performed essentially as described by Balk and Mescher (34). All manipulations were performed in RHS medium or in modifications of this medium. RHS medium consists of RPMI 1640 without $NaHCO_3$ (Gibco) supplemented with salts to yield 127 mM NaCl, 1.8 mM $MgCl_2$, and 1.3 mM $CaCl_2$, 10 mM Hepes, pH 7.2–7.4, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% heat-inactivated fetal calf serum. RHS-EGTA medium is RHS medium with 5 mM EGTA (ethyleneglycol-bis-(B-amino-ethyl ether) *N,N'*-tetra-acetic acid, a Ca^{++} chelator) (Sigma Chemical Co., St. Louis, MO). RHS-Dextra medium is RHS medium with 10% wt/vol dextran T-500 (Pharmacia Fine Chemicals).

Cloned CTL and ^{51}Cr-labeled target cells were washed once in RHS-EGTA medium and resuspended at 10 × 10^6/ml (CTL) or 1 × 10^6/ml (targets) in RHS-EGTA. Step 1: conjugate formation. Typically, 2 ml of CTL and 1 ml of targets were placed in a round-bottom 12 × 75 mm tube (Falcon #2058) and centrifuged for 5 min at 250 *g* at 23°C. Step 2: conjugate dissociation. 1,700 μl of supernatant was removed and the cell pellet gently resuspended in the remaining medium. 0.1-ml aliquots of CTL-target conjugates were then transferred to 16 × 125 mm tubes (Falcon #3033) containing 0.2 ml medium RHS-EGTA alone or in medium containing various concentrations of unlabeled competitor B cells. These tubes were then secured upright in a test tube rack that was fixed on an orbital shaker (33-cm platform, Arthur H. Thomas, Philadelphia, PA) and shaken at a setting of 6–6.5 to keep cells in suspension. Step 3: conjugate measurement. At varying times tubes were removed from the shaker and duplicate 0.1-ml aliquots per sample were transferred to each of two 12 × 75 mm tubes with caps (Falcon #2058) containing 2 ml RHS-dextran. The tubes were vortexed and incubated upright in a waterbath for 4 h at 37°C to permit lysis of bound targets. 2 ml of cold phosphate-buffered saline were then added to each tube, the tubes were vortexed, centrifuged at 1,600 *g* for 10 min, and 3 ml of supernatant (¾ of total) removed for measurement of ^{51}Cr release. Both supernatant and pellet for each sample were counted and the percent total release calculated by the formula: [supernatant cpm × 1.33/(supernatant cpm + pellet cpm)] × 100. The specific release was calculated as (% total release − % spontaneous release)/(95% − % spontaneous release) (26).

Indirect Immunofluorescence and Flow Microfluorometry (FMF). Cell surface antigens were analyzed on CTL clones by indirect immunofluorescence and FMF using a FACS IV cell sorter (B-D FACS Systems, Becton, Dickinson and Co., Sunnyvale, CA) with logarithmic amplification and data collected in 256 channels. For FMF analysis, CTL clones were expanded for 5 d in the presence of specific 6000 R PBL stimulator cells plus 6.7% lectin-free TCGF derived from the MLA 144 gibbon T cell line (35) (a kind gift of Dr. F. Ruscetti, Frederick Cancer Research Center, Frederick, MD). Cells were incubated with saturating concentrations of antibodies (1:1,000 dilution of ascites for antibodies OKT3, OKT4, and OKT8 or 10 μg/ml of anti-Tac) as previously described (36), and stained with a fluorescein-conjugated $F(ab')_2$ preparation of goat anti-mouse IgG (heavy and light chain specific, N.L. Cappel Laboratories, Cochranville, PA). The mean fluorescence intensity of cells in a positive peak was calculated as follows: ϵ (channel no. × no. of cells in channel)/total no. of cells in peak. The percentage of positive cells was determined as

the percentage of cells to the right of the overlap of the negative control curve with the curve obtained with antibody.

Results

Characterization of SB-Specific CTL Clones. The seven SB-specific CTL clones (Table II) utilized in the present study were derived by limiting dilution culture at 0.3 cells per well from three primed lymphocyte populations identical to those used to originally define the SB antigens. The specificity of the clones was confirmed by cytotoxicity testing on panels of B cell lines (Table II). For example, the two clones derived from an SB1-specific bulk population (1.1, 5.5) killed only the two SB1-positive targets among the five B cell lines matched for all known HLA antigens except SB.

All of the clones were analyzed by FMF for the presence of cell surface T3, T4, T8, and Tac antigens by indirect immunofluorescence with the OKT3, OKT4, OKT8A, and anti-Tac antibodies (Table III). All fluorescence profiles appeared unimodal, and gave no indication of discrete subpopulations of cells

TABLE II
SB Specificity of CTL Clones

Clone	Derived from donor combination	Expected specificity	Percent specific lysis of target cells*					
			H9B	K4B	W7B	M14B	PM1B	S11B
1.1	FB6 anti-FB11	SB1	17	14	23	−2	0	0
5.5	FB6 anti-FB11	SB1	27	38	32	1	0	−1
	SB specificities of target:		1,2	1	1,4	3,4	2,3	2,5
	DR specificities of target:		2,3	1,3	2,3	2,3	2,3	2,3
			C5B	H9B	FB6B	FB11B	M14B	
8.1	W7 anti-H9	SB2	24	45	−2	2	0	
8.2	F2 anti-B17	SB2	15	17	−2	3	0	
8.4	F2 anti-B17	SB2	27	49	−1	1	0	
8.6	F2 anti-B17	SB2	9	19	−1	0	−1	
8.8	F2 anti-B17	SB2	67	79	5	−1	−2	
	SB specificities of target:		2,4	1,2	4	1	3,4	
	DR specificities of target:		3,5	2,3	4,4	4,4	2,3	

* All CTL clones were assayed at effector/target (E:T) of 10:1 or 20:1.

TABLE III
Cell Surface Phenotype of CTL Clones

Clone	Percentage of cells binding above background:			
	OKT3	OKT4	OKT8A	Anti-Tac
1.1	96 (100)*	96 (100)*	0	74
5.5	89 (88)	93 (95)	13	86
8.1	89 (99)	89 (96)	7	49
8.2	93 (94)	94 (94)	5	67
8.4	92 (96)	91 (92)	1	49
8.6	94 (108)	94 (113)	0	40
8.8	96 (116)	96 (109)	0	73

* Number in parentheses are the mean fluorescence intensity values of the positive peak.

within the clonal populations (data not shown). All clones were clearly T3⁺, T4⁺, T8⁻, except that clone 5.5 was highly autofluorescent and gave background staining of 5–15% positive with all antibodies tested, including OKT8A. No significant differences were noted between the clones in the level of expression of the T3 or T4 molecules as measured by mean fluorescence intensity. The surface expression of the Tac antigen was variable among the clones. The majority of the cells in a clonal population were Tac⁺, but there was a wide spectrum of very dull and very bright cells (data not shown).

Inhibition of CTL Activity by Monoclonal Antibodies. The panel of SB-specific CTL clones was assayed for susceptibility to inhibition of CTL activity by the monoclonal antibodies OKT3, OKT4A, OKT4F, and anti-Tac (Fig. 1). Cytotoxicity was assayed on M16B targets that express both SB1 and SB2 antigens. Marked differences in susceptibility to inhibition were observed among the clones. Although both SB1-specific clones (1.1 and 5.5) were inhibited by OKT4A and OKT3, 1.1 was completely inhibited by a concentration of OKT4A (1 μg/ml) that resulted in little blocking activity on clone 5.5. This difference for inhibition by OKT4A was paralleled by a similar difference for inhibition by

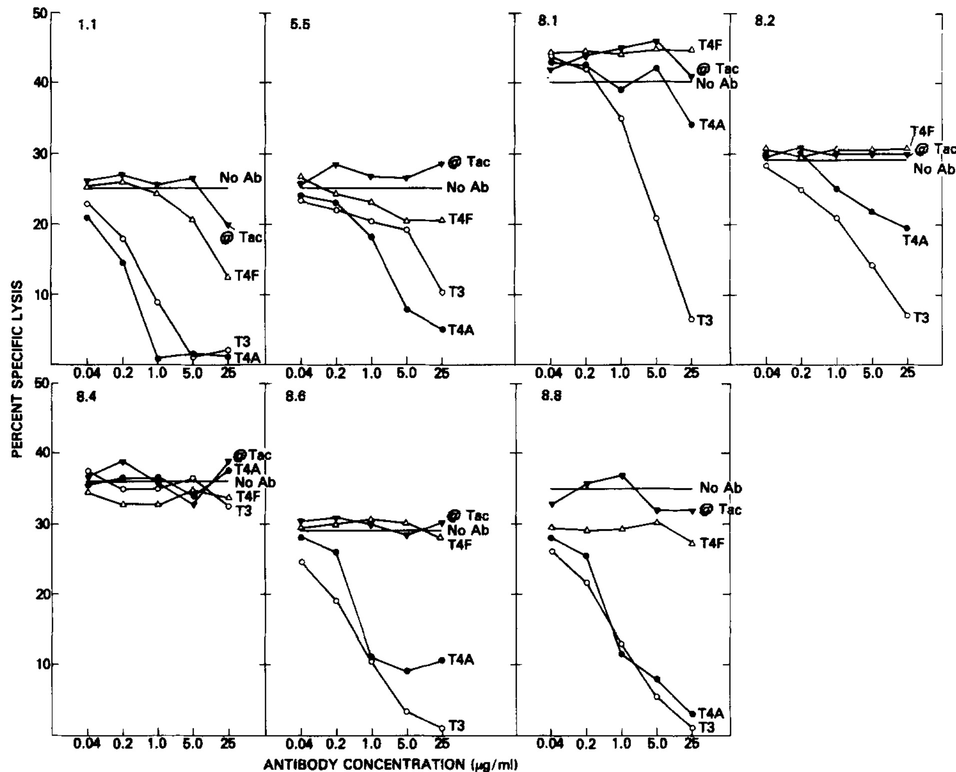

FIGURE 1. SB-specific CTL clones differ in their susceptibility to inhibition by anti-T3 and anti-T4 antibodies. SB-specific CTL clones (50 μl) were preincubated for 20 min with 50 μl of the indicated concentrations of monoclonal antibodies anti-Tac (▼), OKT3 (○), OKT4A (●), OKT4F (△), or medium alone (—No Ab). ^{51}Cr-labeled M16B target cells (50 μl) were then added and lysis was measured 2–4 h later. E:T = 10:1 or 20:1.

OKT3. Antibody OKT4F, which binds to an epitope on the T4 molecule distinct from OKT4A, had marginal blocking activity on any of the clones, as did anti-Tac. The SB2-specific clones also differed widely in their susceptibility to blocking: clone 8.4 was completely refractory to inhibition by any of the antibodies at concentrations as high as 25 μg/ml. Clones 8.6 and 8.8 were strongly inhibited by OKT4A at 5–25 μg/ml, whereas these concentrations of OKT4A produced little inhibition of 8.1 or 8.2.

Assay for Tightness of Binding of CTL to Targets. The differences in the susceptibility of $T4^+$ SB-specific CTL to inhibition by anti-T4 and anti-T3 antibodies could be due to differences in the affinity of the CTL for targets. Classical measurements of affinity performed on molecules in solution cannot be used to assess the affinity of interaction between cells. As an alternative, we have estimated how tightly the CTL are bound to targets by determining the dissociation of CTL–target cell conjugates in the presence of an excess of unlabeled competitor cells identical to the targets. The procedure for making this measurement is essentially as described by Balk and Mescher (34). The assay can be viewed as three distinct steps: (*a*) formation of conjugates; (*b*) dissociation of conjugates; and (*c*) enumeration of remaining conjugates. The first two steps are performed in Ca^{++}-free medium to allow normal binding but to prevent delivery of the lethal hit. Conjugate formation is optimized by pelleting effectors and labeled targets. The dissociation phase is in suspension with continuous mixing in the presence of graded doses of competitor cells identical to the target but unlabeled. The number of labeled targets remaining in conjugates after dissociation (and/or exchange with competitor cells [34]) is estimated by transferring the cells to Ca^{++}-containing medium at 37°C to allow delivery of the lethal hit in existing conjugates, but in a semi-solid medium to prevent formation of new conjugates.

The ability of cold targets to reverse functional CTL–target cell conjugates is illustrated by the results in Fig. 2. Clone 8.8 cells were mixed with ^{51}Cr-labeled M16B targets, centrifuged, and diluted either with medium alone or unlabeled M16B cells. Aliquots of these mixtures were either transferred immediately to the dextran medium to measure the control level of lysis by functional CTL–target cell conjugates (time 0), or they were cultured in suspension for 1, 2, or 4 h before transfer to dextran. Culture in suspension with medium alone did not significantly change the level of ^{51}Cr release, indicating that CTL-target cell conjugates could not readily dissociate in the absence of competing cells. Addition of cold M16B cells induced complete inhibition of ^{51}Cr release by 2 h of culture, indicating that functional CTL-^{51}Cr-labeled target cell conjugates could be completely dissociated by competitor cells. Addition of competitor cells in the dextran (lytic) phase had no effect, since there is no significant difference between the lysis obtained at time 0 with or without added M16B cells. These results are very similar to those originally described by Balk and Mescher (34). All subsequent assays were performed by incubating the formed conjugates for 3 h in medium or medium with unlabeled competitor cells, at which point dissociation is readily observed.

Studies were then performed to analyze the ability of graded doses of competitor cells to induce dissociation/exchange of conjugates formed by each of the

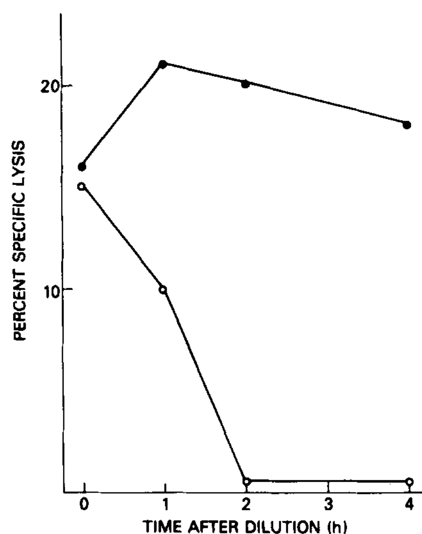

FIGURE 2. Kinetics of reversal of functional CTL conjugates. Conjugates were formed between clone 8.8 and M16B ^{51}Cr-labeled targets by centrifugation. Conjugates were then diluted in either media alone (●) or media containing unlabeled M16B cells at 20 × 10^6/ml (○) and shaken for various times at 23°C. Aliquots were then transferred to RHS-dextran medium for 4 h at 37°C to measure ^{51}Cr release from remaining conjugates.

seven clones. A typical study comparing two clones (8.1, 8.8) is shown in Fig. 3. $SB2^+$ cold targets M16B are able to induce dissociation of ^{51}Cr-labeled M16B-8.8 conjugates (measured as a decrease in subsequent ^{51}Cr release), but not M16B-8.1 conjugates. The number of cold M16B cells required to inhibit 50% of the control lytic activity of clone 8.1 is at least 10-fold greater than that required to inhibit the same amount of lytic activity of clone 8.8. The competition is specific, as originally described (34), since little or no dissociation of functional conjugates is induced by competing cells autologous to the responder (which lack the SB2 antigen).

Comparison of CTL Clones for Tightness of Binding Targets and Susceptibility to Inhibition by Anti-T4 and Anti-T3 Antibodies. To determine whether there is a correlation between susceptibility to inhibition by OKT3/OKT4A and tightness of cell interaction (estimated by the dissociation studies), the titration data in each system were reduced to a single value representing the number of cells or quantity of antibody required to reduce cytotoxic activity to 50% of the control value. The values were then tabulated and compared (Table IV). The results of experiment 1 demonstrate that clones 8.1 and 8.8 differ as follows: (*a*) 8.1 requires greater than 10-fold more competitor cells to induce conjugate dissociation than does 8.8; and (*b*) 8.1 requires at least 50-fold more anti-T4 and 10-fold more anti-T3 antibody to inhibit cytolytic activity than does 8.8. Similar comparisons with different clones are shown in experiments 2–4. In each paired comparison, the clone that requires the most competitor cells to induce conjugate dissociation requires the most anti-T4 and anti-T3 antibody to inhibit cytotoxicity. A comparison of two clones that require almost identical quantities of anti-T3 and anti-T4 antibodies to inhibit cytotoxicity (clones 1.1 and 8.8 in experi-

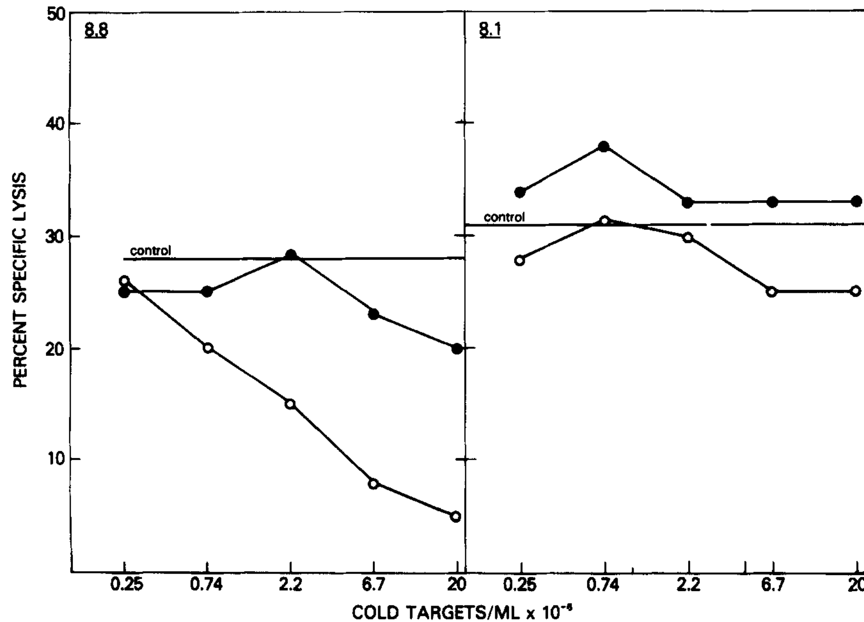

FIGURE 3. Comparison of clones 8.8 and 8.1 for tightness of binding to specific target cells. Conjugates were formed between clones 8.8 or 8.1 and ^{51}Cr-labeled M16B cells by centrifugation. Conjugates were then diluted in medium alone (—control), various concentrations of unlabeled SB2-negative F2B cells (●), or unlabeled SB2-positive M16B cells (○), and shaken for 3 h at 23°C. Aliquots were then transferred to RHS-dextran medium for 4 h at 37°C to measure ^{51}Cr release from remaining conjugates.

ment 5) demonstrates that they require virtually identical concentrations of competitor cells for dissociation in the conjugate reversal assay. These results indicate that within this panel of SB-specific CTL clones, there is an inverse correlation between tightness of target cell binding and susceptibility to inhibition by anti-T4 and anti-T3 antibodies.

Dissociation of Functional CTL–Target Cell Conjugates by Anti-T4 Antibodies. If the T4 molecule is involved functionally in target cell binding, then anti-T4 antibodies might induce dissociation of conjugates. To address this possibility experimentally, the conjugate dissociation assay was performed as described in Fig. 3 and Table IV with the following modification: anti-T4 and control anti-Tac antibodies were substituted for unlabeled competitor cells in the dissociation phase. Results of such an assay with five clones are given in Table V. OKT4A and OKT4B, but not anti-Tac, appeared to partially induce dissociation of ^{51}Cr-labeled targets from four clones, although this was not very striking for clone 8.1. To assure that the blocking observed was due to its effect during the dissociation (step 2) rather than the lethal hit stage (step 3), the controls were performed with addition of an equivalent amount of antibody at step 3 (Table V, right-hand column). Inhibition by anti-T4 antibodies at step 3 was negligible.

Clone 8.4 appears to be the "highest affinity" clone in the panel, since it is the most resistant to antibody-mediated blocking of cytotoxicity and conjugate dissociation induced by competitor cells. Clone 8.4 was also unique in that OKT4A could not induce dissociation of 8.4-M16B conjugates when the assay

TABLE IV

Comparison of Number of Unlabeled Specific Competitor Cells Required to Reverse CTL-Target Conjugates with Amount of OKT4A and OKT3 Required to Inhibit Cytotoxicity

Expt. no.	Clone	Amount required to reduce cytotoxicity by 50%:*		
		M16B competitor cells × 10^{-6}/ml*	OKT4A µg/ml‡	OKT3 µg/ml‡
1	8.1	>20	>25	5.0
	8.8	2.6	0.5	0.5
2	8.2	16	>25	5.0
	8.6	1.3	0.6	0.6
3	1.1	6.7	0.6	0.6
	5.5	>20	2.0	18
4	8.4	>20	>25	>25
	8.6	6.7	0.6	0.6
5	1.1	5.4	0.6	0.6
	8.8	4.2	0.5	0.5

* Each cloned CTL was reacted with ^{51}Cr-labeled M16B targets and CTL-target conjugates were reversed by dilution and incubation with graded numbers of unlabeled M16B as in Fig. 4. The number of unlabeled M16B competitor cells required to reduce cytotoxicity by 50% (i.e., percent specific lysis in the absence of competitor cells/2) was then interpolated from the titration curve.

‡ Values are the amount (in µg/ml) of antibody required to reduce cytotoxicity by 50% (relative to lysis in the absence of antibody). Values are interpolated from the antibody titration curves in Fig. 1.

was performed under standard conditions (23°C) (Table V). As one approach to determine whether anti-T4 antibodies could induce conjugate dissociation under different experimental conditions, the temperature of the conjugate formation and dissociation steps was lowered to 4°C. The overall level of conjugate formation was lower at 4°C, as revealed by the lower lysis in medium only (25% vs. 60%), but at 4°C marked dissociation was induced by OKT4A (Table V).

Discussion

Previous studies have demonstrated that antibodies against the T4 molecule can inhibit antigen-specific functions of T4$^+$ cells (10, 11, 16–19). It has been hypothesized that this antibody-mediated inhibition results from the disruption of binding of T cells to targets, perhaps by interference with the T4 molecule binding to a nonpolymorphic epitope on class II MHC molecules (10, 16, 24). However, considerable heterogeneity among SB-specific CTL populations has been observed in their susceptibility to inhibition by anti-T4 antibodies (10). SB2-specific bulk culture–derived CTL were much more resistant to anti-T4 inhibition than were analogous CTL populations specific for SB1,3, or 4 (10). It was proposed that these observed differences might reflect differences between SB-specific T cell clones in their affinity for antigen (10). The present study demonstrates that: (*a*) there is considerable clonal heterogeneity in the ability of SB-specific CTL clones to be inhibited by anti-T4 as well as anti-T3 antibodies; (*b*) there is also marked heterogeneity in the tightness of binding of CTL clones

TABLE V
Anti-T4 Antibodies Can Induce Partial Dissociation of Functional CTL-Target Cell Conjugates

Clone	Antibody	Phase of antibody addition*		
		Conjugate dissociation (step #2)		Lysis (step #3)
		4°C	23°C	
1.1	None		41‡	41‡
	Anti-Tac		40	40
	OKT4A		27	40
	OKT4B		25	44
5.5	None		42	42
	Anti-Tac		43	40
	OKT4A		21	36
8.1	None		58	58
	Anti-Tac		58	60
	OKT4A		46	54
8.2	None		55	55
	Anti-Tac		49	53
	OKT4A		33	46
8.4	None	25	60	60
	Anti-Tac	19	56	56
	OKT4A	9	61	61

* M16B ^{51}Cr-labeled targets were mixed with CTL clones, centrifuged at 4°C or 23°C, and diluted in either RHS-EGTA medium or antibodies diluted in the same medium to 25 μg/ml. During the conjugate dissociation phase, the mixture of cells with and without antibodies were kept in suspension culture for 3 h at 23°C, except for clone 8.4 which was cultured at either 4°C or 23°C. An aliquot of this mixture was then transferred to 2 ml of 10% dextran medium and lysis of bound targets was allowed to proceed at 37°C for 4 h. For control of antibody inhibition of lysis in the dextran phase of the assay, an aliquot of antibody equivalent to that transferred with the conjugate mixture was included in the 10% dextran medium.
‡ % specific lysis after 4 h in 10% dextran medium.

to targets as estimated by CTL–target cell dissociation in the presence of competitor cells; (*c*) clones that are most susceptible to antibody-mediated inhibition are those that bind targets less tightly, and vice versa; and (*d*) antibody to the T4 molecule not only can block killing but can induce dissociation of preformed CTL–target cell conjugates.

Antibodies that inhibit cytolysis could block at any stage of the complex of events in cell-mediated cytotoxicity. This process can be partitioned into at least two steps: 1) Ca^{++}-independent binding and conjugate formation; and 2) Ca^{++}-dependent delivery of the lethal hit (37–39). The present study indicates that the T4 molecule participates in the binding phase, since anti-T4 antibodies can disrupt functional CTL–target cell conjugates. This role for T4 molecules may be analogous to that proposed for the T8/Leu-2 molecule in humans and the

Lyt-2 molecule in mice. Both anti–Leu-2a (14) and anti–Lyt-2 (40) antibodies were able to inhibit the binding phase, but not the lytic phase of CTL-mediated cytolysis. Results from two different approaches are consistent with a role for T4 in T cell activation and/or binding. First, anti-L3T4 antibody in mice blocks binding of antigen-specific L3T4$^+$ T cell hybridomas to antigen-pulsed antigen-presenting cells (23). Second, anti-T4 antibodies in man (16–19) and the anti-L3T4 antibody in mice (23) block antigen-specific proliferation.

Marked differences were observed in the SB-specific CTL clones studied in their susceptibility to inhibition by anti-T4 antibodies. Similar observations were made for the susceptibility of Lyt-2$^+$ CTL clones to inhibition by anti–Lyt-2 antibodies (6, 25). Such clonal heterogeneity was postulated to reflect differences in the affinity of T cell clones for antigen (6, 10, 25). Perhaps the most suggestive evidence for this hypothesis was that the specific reactivity of an anti-Moloney sarcoma virus plus H-2^b CTL clone was resistant to inhibition by anti–Lyt-2 antibody, while the allogeneic cross-reactivity of this same clone on uninfected H-2^d targets was inhibited by anti–Lyt-2 antibody (25). To test this hypothesis regarding affinity, we have assessed differences between clones in their tightness of binding to targets. The assay chosen to quantitate tightness of binding measured the ability of cold targets to induce dissociation of preformed conjugates. Balk and Mescher (34) used this assay system to demonstrate that target cell binding to CTL was a reversible process, and that exchange could occur between bound and unbound targets (34). Using this assay, we observed marked heterogeneity in the number of unbound competitor cells required to induce dissociation of bound target cells from individual CTL clones. Although the binding events are undoubtedly complex, we would expect that differences between the affinities of the antigen-specific receptors of the different clones would be the most important factor that contributes to differences in the tightness with which CTL bind targets. However, other differences, such as cell surface density of antigen-specific receptors or density of cell adhesion molecules (see below) or even differences in lateral mobility of those molecules could also contribute to differences in the tightness of target cell binding. To date no evidence has been reported for a structural polymorphism of T3 or T4 molecules on different T cell clones, and we have noted no significant differences in the density of the T3 or T4 molecules on the seven clones in the present study (Table III) (such differences in level of expression of L3T4 have been detected on mouse CTL clones by cell sorter analysis [23]).

Collectively the available data suggest that the T4 molecule is one of a number of cell adhesion molecules on human T lymphocytes. The high frequency of T4 expression on class II MHC-reactive T cells prompted us and others (10, 16, 24) to postulate that the function of the T4 molecule is to bind to a nonpolymorphic epitope on class II MHC molecules. Such a binding function could serve to focus class II MHC molecules onto the surface of T cells and also to provide a stabilizing function for the interaction of low affinity T cells with antigen-bearing cells. The observation that a "high affinity" clone such as 8.4 can be dissociated from bound targets by anti-T4 antibodies at 4°C but not at 23°C indicates that the T4 molecule is providing a binding function even on high "affinity" clones. In order to detect the functional involvement of the T4 molecule on "high affinity" clones,

it is necessary to artificially lower the apparent avidity of the CTL-target interaction by lowering the binding temperature. It remains to be seen whether this indication of T4 function in such a clone will be irrelevant to its function under physiologic conditions or whether it accurately reflects the importance of T4 molecules to all T4-positive clones.

What is the role of the T3 molecule in T cell–mediated cytotoxicity? The correlation between the doses of anti-T3 and anti-T4 antibodies required to inhibit cytotoxicity by clones with differing "affinity" could suggest that T3, like T4, acts at the binding phase. However, evidence from two different studies indicate that anti-T3 antibody cannot block conjugate formation (14, 15), or reverse preformed conjugates (15), but can inhibit a post-adhesion step in cytolysis (15). Work in progress on our clones also indicates that anti-T3 can inhibit at the post-adhesion step, which complicates interpretation of the partial inhibition seen with addition at the dissociation step. Meuer et al. (41) have demonstrated that the T3 molecule co-modulates with the antigen-specific receptor and suggested that this molecule is involved in T cell triggering. It is therefore possible that the inhibitory effects of anti-T3 antibodies could be due to indirect effects on the closely associated antigen-specific receptor (41). Thus, T4 and T3 may be operative in different T cell functions. Perhaps inhibition of both cell adhesion (anti-T4) and of triggering (anti-T3) may be subject to affinity-dependent differences between clones.

At least four different molecules have now been defined on human T cells that are distinct from the antigen-specific receptor and appear to be involved in T cell binding of antigen-bearing cells: T4 on class II–reactive cells (10, 11, 16–19), T8 on class I–reactive cells (8, 11, 14, 16, 17, 19), LFA-1 (42), and LFA-2 (42). The general picture that is emerging from all of these studies is that the binding of a target cell or antigen-presenting cell by a T cell involves a collection of molecular interactions. It is unclear how interactions of these diverse molecules are orchestrated into the finely regulated, highly specific response characteristic of T cell clones. Perhaps there is some complex additivity of their affinities for the target cell; perhaps there is some loose physical association of several molecules into a receptor complex; or perhaps there are intracellular mechanisms for coordinating signals that they transduce. Novel approaches will be required to elucidate these complex possibilities.

Summary

The present study examines the potential role of the T4 molecule in functional cell-cell interactions between target cells and human cytotoxic T lymphocyte (CTL) clones that are specific for HLA class II alloantigens encoded by the SB locus. There were marked differences (greater than 30-fold) between the seven SB-specific clones studied with respect to their susceptibility to inhibition by anti-T4 as well as anti-T3 antibodies. We wished to test the hypothesis that such variation among the clones would be due to differences in clonal "affinity" for antigen. To quantitate differences among the CTL clones in the tightness with which they bind target cells, the clones were analyzed using a previously published assay of susceptibility of CTL–target cell conjugates to dissociation in the presence of unlabeled targets. The results revealed that the clones that were most

susceptible to inhibition by anti-T4 and anti-T3 were the weakest target cell binders, and vice versa. Anti-T4 antibody could partially induce dissociation of functional CTL–target cell conjugates in the absence of any added cold targets. For the "highest affinity" clone such anti-T4 antibody-induced dissociation could be observed at 4°C but not 23°C. These results indicate that the T4 molecule is functionally involved in target cell binding by CTL, and raise the possibility that although it is easiest to demonstrate the function of the T4 molecule in "low affinity" clones, that function may also be operative in the "high affinity" clones.

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