

# T LYMPHOCYTES SPECIFIC FOR IMMUNOGLOBULIN ALLOTYPE

## II. Cloned Igh-1<sup>b</sup>-specific Cytotoxic T Cells\*

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Despite much effort, the structural features of the surface molecules responsible for antigen recognition by thymus-derived (T) cells remain unclear (1, 2). Progress in this area will in part depend on the availability of homogeneous T cells from which to isolate and purify T cell receptor molecules in quantities sufficient to characterize them. With the advent of recent technology, the development of T cell clones (3), and the description of mitogen-induced T cell growth factors (TCGF; 4, 5),<sup>1</sup> it has become possible to generate and maintain clones of antigen-specific T cells indefinitely in culture in appreciable numbers.

In this communication, we provide a preliminary characterization of long-term-cultured lines and clones of cytotoxic T lymphocytes (Tc) with specificity directed to determinants of the Igh-1<sup>b</sup> immunoglobulin (Ig) allotype. These cell lines and clones were initiated by repeated stimulation of Igh-1<sup>b</sup>-immune BALB/c spleen cells with an Igh-1<sup>b</sup>-producing myeloma (6), and subsequent maintenance in vitro in medium supplemented with mitogen-induced growth factors.

These cloned allotype-specific Tc are unique in that their cytotoxic activity can be inhibited by soluble antigen, i.e., Igh-1<sup>b</sup>. In view of the available extensive knowledge of the Igh-1<sup>b</sup> molecule, the antigen in this system, it seems likely that these allotype-specific Tc will prove to be useful starting material for both detailed studies of killer T cell functions and for molecular characterization of their receptors.

### Materials and Methods

All of the techniques have been described in the accompanying paper (6) with the following exceptions:

*Long-Term Lines and Clones of Tc Cells.* Procedures similar to those described by others (7-9) were employed to initiate continuous long-term lines of killer cells. Spleen cells from BALB/c mice immunized with Igh-1<sup>b</sup> myeloma proteins were restimulated in vitro three times in succession at 10-14-d intervals. The number of responder spleen cells was  $5 \times 10^6$ ,  $1 \times 10^6$ , and  $0.2 \times 10^6$  cells/ml for the primary, secondary and tertiary cultures. After the third restimulation, the remaining cells were cultured ( $10^4$ - $10^5$  cells/ml) without further antigen in medium supplemented with 30% (vol:vol) TCGF (supernate from BALB/c spleen cells cultured for 36-

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<sup>1</sup> Abbreviations used in this paper: LPS, lipopolysaccharide; MHC, major histocompatibility complex; Tc, cytotoxic T cells; TCGF, T cell growth factor.

48 h with 5  $\mu\text{g}/\text{ml}$  concanavalin A). Clones were established by limiting dilution procedures in microtiter plates using syngeneic feeder cells ( $2 \times 10^4$  peritoneal cells/well) in medium supplemented with TCGF (30%, vol:vol). All clones were selected from plates with  $<10\%$  positive wells. The cloning efficiency from primary cultures was 1–10%; in the preparation of subclones it was  $>60\%$ .

*Miscellaneous Techniques.* Ig were radiolabeled with  $^{125}\text{I}$  by the chloramine T procedure (10) to specific activities of  $2 \times 10^5$ – $12 \times 10^6$  cpm/ $\mu\text{g}$  of protein.

Anti-Thy-1.2 antiserum was prepared by repeated immunization of AKR/J mice with C3H/J thymus cells. For removal of T cells, 0.2 ml of this antiserum was added to  $2.5 \times 10^7$  cells. After 30 min on ice, the cells were washed twice and resuspended in 2 ml guinea pig serum (1:7 dilution), as a source of complement, and incubated for 45 min at  $37^\circ\text{C}$ .

## Results

*Derivation and Lytic Potency of Allotype-specific Tc Cell Lines and Clones.* Long-term BALB/c anti-Igh-1<sup>b</sup> Tc cell lines were initiated as described above and tested for lytic activity at various times against CB101 targets. Fig. 1 illustrates the time-course of the cytolytic activity of three such independent Tc lines. Without cloning, many of the Tc lines eventually lose their cytolytic activity, as seen with the T2 line. However, in some cases it is possible to maintain cytolytic activity for long periods of time without cloning, as with the T4 cell line.

At 1.5 mo after initiation, the T10 line was cloned by limiting dilution, and two cytotoxic clones (T10.1A and T10.1B) were selected. Then at 3.5 mo, T10.1B was subcloned, yielding the clones T10.1B.2A, T10.1B.2C, and T10.1B.2D. Fig. 2 illustrates the relative potency of these lines and clones compared with primary Tc populations, immune spleen cells that have been restimulated in culture only once. It is evident that Tc lines and clones are 30–100 times more potent than primary Tc populations.

These long-term cell lines and clones have been identified as T cells by their sensitivity to anti-Thy-1.2 and complement treatment (Table I).

*Lytic Specificity.* The functional specificity of these Tc clones is depicted in Fig. 3. These T cells exhibit the same specificity as primary Tc populations (6). That is, they are lytic for CB101 cells and relatively nonlytic for CB101NP, (non-Igh-1<sup>b</sup>-producing variant), U10 (Igh-1<sup>a</sup>), and S10P (Igh-4<sup>b</sup>) target cells and mitogen-induced BALB/c blast cells. The T10.1B.2C clone is distinct from its sister subclones in that it is generally more lytic for all targets tested.

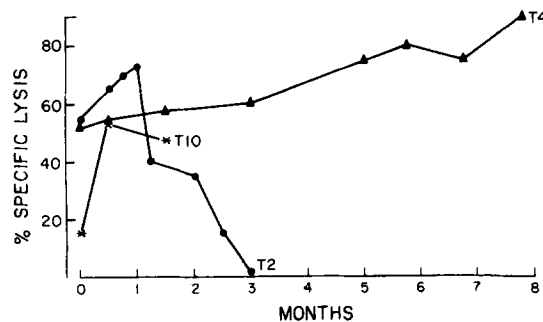


FIG. 1. Lytic activity (effector:target ratios = 20:1) of three independently derived, uncloned lines of allotype-specific Tc.

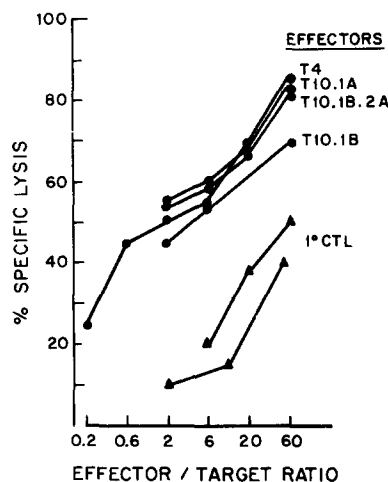


FIG. 2. Lytic potency of three clones from the T10 line, and of the T4 line compared with primary cultures of allotype-specific Tc.

TABLE I  
*Abolition of Cytotoxic Activity in Long-Term Allotype-specific Tc Lines with Anti-Thy-1.2 and Complement*

Tc effectors	Percent specific lysis of CB101 targets after treatment of effectors with*	
	Complement	Anti-Thy-1.2 + Complement
T4	71	11
T10.1B	60	3

\* Before treatment with antiserum and/or complement, the effector:target cell ratio was adjusted to 40:1 for T4 and 10:1 for T10.1B. No adjustments were made in cell number after treatment.

The specificity of these Tc clones with respect to major histocompatibility complex (MHC) restriction is shown in two experiments in Fig. 4. Cloned Tc and primary cultures of anti-Igh-1<sup>b</sup> Tc were tested on <sup>51</sup>Cr-labeled CB101 (*Igh-1<sup>b</sup>, H-2<sup>d</sup>*), CB101NP (*H-2<sup>d</sup>*), and B79 (*Igh-1<sup>b</sup>, H-2<sup>b</sup>*) target cells. The latter is a plasmacytoma, BEPC79, derived from CXBE mice by Dr. M. Potter, National Cancer Institute. As expected, CB101NP targets were not lysed significantly; however, the T10.1B.2C clone displayed appreciable lytic activity on B79, the Igh-1<sup>b</sup> expressing *H-2<sup>b</sup>* target cell.

*Inhibition of Cloned Allotype-specific Tc by Soluble Igh-1<sup>b</sup>.* In an effort to define better the specificity of these allotype-specific Tc clones, the effect of preincubation of the T10.1B.2A clone with various Ig preparations was examined. As demonstrated in Table II, affinity-purified Igh-1<sup>b</sup> protein dramatically reduces the lytic capacity of T10.1B.2A without affecting an unrelated allo-MHC-specific Tc population. This effect is Ig allotype-specific, because Igh-1<sup>a</sup>, Igh-4<sup>b</sup>, and Igh-4<sup>a</sup> did not inhibit lysis. <sup>125</sup>I-labeled proteins were found to be far more efficient in causing this effect, whereas much higher concentrations (1–10 mg/ml) of unlabeled Igh-1<sup>b</sup> were required to achieve the same results; and labeled Igh-1<sup>b</sup> preparations did not inhibit the lytic activity of primary cultures of anti-Igh-1<sup>b</sup> killer cells (Table III).

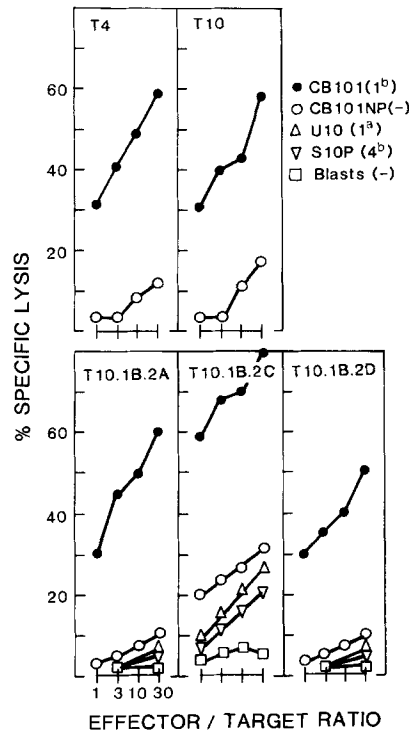


FIG. 3. Lytic specificity of three clones and two lines of allotype-specific Tc tested on a variety of different targets.

*Mechanism of Igh-1<sup>b</sup> Inhibition of Tc Function.* Efforts were made to quantitate the direct binding of soluble Ig with these Tc clones. Radiolabeled Igh-1<sup>a</sup> and Igh-1<sup>b</sup> preparations (30–100  $\mu\text{g}/\text{ml}$ ) were incubated with T10.1B.2A cells for 60 min at 37°C. The cells were then washed extensively and the amount of bound Ig was determined. The cytotoxic activity of the T cells after this treatment was then assessed. Table IV demonstrates that there was no appreciable difference in the binding of Igh-1<sup>b</sup> and Igh-1<sup>a</sup> even though Igh-1<sup>b</sup> markedly inhibited lytic activity against Igh-1<sup>b</sup> targets.

Table V explores the effect of temperature dependency of the lytic inhibition with soluble Ig proteins. For these experiments, killer populations were incubated with or without Igh-1<sup>b</sup> protein at 4 or at 37°C for 40 min, washed, and then incubated with <sup>51</sup>Cr-labeled CB101 target cells. The results show that the inhibition of lysis of Igh-1<sup>b</sup> targets with soluble Igh-1<sup>b</sup> proteins requires preincubation at 37°C.

One explanation for the mechanism by which soluble Igh-1<sup>b</sup> inhibits the lytic activity of the T10.1B.2A clone is that the Ig molecule serves to bridge two Tc cells, causing one to lyse the other. This possibility of “autolysis” is supported by experiments described in Table VI. T10.1B.2A killer cells were radiolabeled with <sup>51</sup>Cr and then incubated for 4 h with various Ig at 37°C. Supernates were then harvested, and the percent of <sup>51</sup>Cr released was determined. As can be seen, Igh-1<sup>b</sup> induced significant <sup>51</sup>Cr release from T10.1B.2A cells without affecting lipopolysaccharide (LPS) blasts.

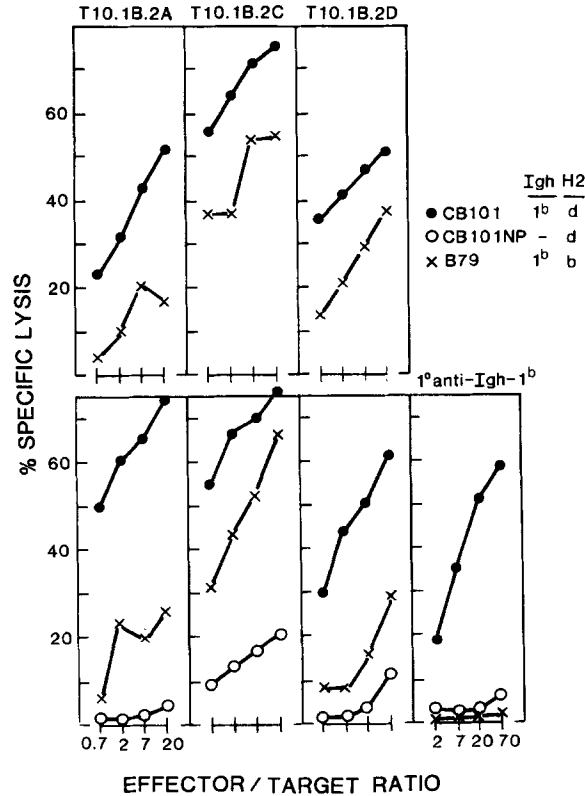


FIG. 4. Top panel: comparison of lytic specificity of allotype-specific clones on Igh-1<sup>b</sup> targets expressing the H-2<sup>d</sup> (self) or H-2<sup>b</sup> (allo) MHC haplotype. Bottom panel: this experiment was repeated and included the use of CB101NP target and primary cultures of allotype-specific Tc.

Again, this effect was allotype-specific because Igh-1<sup>a</sup> and Igh-4<sup>a</sup> did not cause lysis of anti-Igh-1<sup>b</sup> killer cells.

*Suppression of Igh-1<sup>b</sup> Expression In Vivo with an Allotype-specific Tc Line.* Experiments similar to those in the preceding paper with primary cultures of allotype-specific Tc populations were conducted to determine whether a long-term cultured line would inhibit the expression of Igh-1<sup>b</sup> allotype in vivo. The data presented in Table VII show that Tc cells of the T4 line, maintained in the absence of antigen in culture for 8 mo and showing 63% specific lysis on CB101 target cells, were able to inhibit expression of the Igh-1<sup>b</sup> allotype over a 3-4-wk period by cotransferred C.B-17 spleen cells in BALB/c mice. The suppression in this experiment was specific; these animals continued to express the C.B-17 Igh-4<sup>b</sup> allotype.

## Discussion

*Derivation of Tc Lines and Clones.* The long-term Tc lines described here were established and maintained by techniques similar to those described by others. This involved three to four sequential antigen stimulations in culture, followed by further maintenance without antigen in medium supplemented with concanavalin A-induced TCGF. The cytotoxic activity of independently derived T cell lines varied greatly and

TABLE II  
Inhibition of Allotype-specific Tc Activity with Soluble Ig\*

Ig	Inhibitor concentration‡	Labeled	Percent specific lysis after treatment of Tc effectors					
			T10.1B.2A				BALB/c anti-B6	
			Experiment	1	2	3	4	1
PBS			26	62	42	43	48	55
Igh-1 <sup>b</sup>	(30 µg/ml)	+	3	16	20			52
	(60 µg/ml)	+			4	10	39	
	(90 µg/ml)	-			45			
	(9 mg/ml)	-			4	5	34	
Igh-1 <sup>a</sup>	(50 µg/ml)	+	21	50			40	50
Igh-4 <sup>b</sup>	(21 µg/ml)	+	24	62	46	50	44	56
Igh-4 <sup>a</sup>	(40 µg/ml)	+		60	50			

\* T10.1B.2A anti-Igh-1<sup>b</sup> Tc and BALB/c anti-B1/6 mixed lymphocyte culture cells were incubated with the various Ig preparations in the concentrations indicated for 60 min at 37°C. They were washed twice, then tested for residual lytic activity on CB101 cells or on B6 concanavalin A blasts. The effector:target ratios in the various experiments were 10-40:1.

‡ Inhibitors used were: phosphate-buffered saline (0.5% bovine serum albumin); chromatographically purified Igh-1<sup>a</sup>, Igh-4<sup>a</sup>, and Igh-4<sup>b</sup> myeloma proteins; Igh-1<sup>b</sup> affinity-purified ascites fluid from R3-367, an anti-(3-nitro-4-hydroxyphenyl)acetyl-producing hybridoma.

TABLE III  
Inhibition with Soluble Ig of Lytic Activity by Primary and Cloned Allotype-specific Tc

Inhibitor Ig*	Percent specific lysis after treatment of effectors	
	T10.1B.2A	Primary anti-Igh-1 <sup>b</sup>
PBS	62	52
Igh-1 <sup>b</sup> (33 µg/ml)	16	48
Igh-4 <sup>b</sup> (50 µg/ml)	62	54

\* <sup>125</sup>I-labeled proteins were used as inhibitors; effector:target cell ratios 20:1 for T10.1B.2A and 60:1 for primary anti-Igh-1<sup>b</sup> Tc; other conditions were as in Table II.

it was not necessarily stable (Fig. 1). It seems likely that the loss of cytotoxic activity involves overgrowth of the cultures with noncytotoxic cells that are apparently less adherent than Tc. To some extent, loss of lytic activity can be minimized by discarding the nonadherent subpopulation. Such a technique was used in these studies to maintain the T4 line (Fig. 1).

Cloned Tc were obtained from long-term lines by limiting dilution techniques. On a cell for cell basis, these Tc clones were 30-100 times more cytotoxic than primary Tc populations (Fig. 2). At the time of these experiments, the T4 lines had been in culture for ~8 mo, and although it was not intentionally cloned, it may have "cloned itself" because it is as cytotoxic as the three clones derived from the T10 line.

*Lytic Specificity.* The T cell lines (T4 and T10), and the clones (T10.1B.2A,

TABLE IV  
*Direct Binding of Soluble Ig to Allotype-specific Tc Cells and Its Effect Upon Cytolytic Activity\**

Experiment	Myeloma protein	Percent of input Ig bound	Percent specific lysis of CB101 target
A	None	—	50
	Igh-1 <sup>b</sup>	0.6	-0.1
	Igh-1 <sup>a</sup>	0.7	60
B	None	—	25
	Igh-1 <sup>b</sup>	0.5	11
	Igh-1 <sup>a</sup>	0.4	26

\* <sup>125</sup>I-labeled myeloma proteins (30–100 µg/ml) were incubated with T10.1B.2B cells for 60 min at 37°C, washed three times, and the percentage bound was determined. The cells were then assayed for the ability to lyse CB101 target cells.

TABLE V  
*Temperature Dependency of the Inhibitory Effects of Soluble Igh-1<sup>b</sup> Upon Allotype-specific Tc Cells*

Incubation of T cells with Igh-1 <sup>b</sup> at*	Percent specific lysis of CB101 targets	
	Experiment 1	Experiment 2
No Ig at 4°C	39	28
No Ig at 37°C	40	26
4°C	38	26
37°C	5	10

\* T10.1B.2A cells (experiment 1,  $5 \times 10^5$ ; experiment 2,  $2.5 \times 10^5$ ) were incubated with 40 µg/ml Igh-1<sup>b</sup> affinity-purified hybridoma protein for 60 min at 4 or 37°C. The cells were washed once and  $1 \times 10^4$  <sup>51</sup>Cr-labeled CB101 cells were added. After a 4-h incubation at 37°C, the percent specific lysis was determined.

TABLE VI  
*Igh-1<sup>b</sup>-induced Autolysis of Anti-Allotype Tc Cells*

Ig*	Percent specific <sup>51</sup> Cr release off‡			
	T10.1B.2A			LPS blasts
	1	2	3	2
Igh-1 <sup>b</sup>	19	5	34	5
Igh-1 <sup>a</sup>	0	2	5	3
Igh-4 <sup>b</sup>	ND	2	8	4

\* Igh-1<sup>b</sup> was affinity-purified R3-367 protein, whereas Igh-1<sup>a</sup> and Igh-4<sup>b</sup> were chromatographically purified U10 and MOPC300 protein, respectively.

‡ T10.1B.2A and LPS blast cells were labeled with <sup>51</sup>Cr and incubated with various Ig (10 mg/ml) for 4 h at 37°C, after which supernates were harvested and the percent specific chromium release was calculated. The spontaneous release of both targets was 10–20%.

TABLE VII  
*Suppression of Igh-1<sup>b</sup> Allotype Expression In Vivo with Cloned Allotype-specific Tc*

T cell population*	Number of animals/total expressing Igh-1 <sup>b</sup> or Igh-4 <sup>b</sup> 3-4 wk after cell transfer	
	Igh-1 <sup>b</sup>	Igh-4 <sup>b</sup>
Normal spleen	5/5	5/5
Immune spleen	0/5	5/5
T4	0/5	5/5

\* Nylon wool-purified T cells ( $5 \times 10^6$ ) from normal or Igh-1<sup>b</sup>-immune BALB/c spleens, or Tc cells ( $1 \times 10^6$ ) from the long-term (8 mo) T4 line were cotransferred with C.B-17 spleen cells ( $5 \times 10^6$ ) into irradiated (550 rad) BALB/c recipients. The presence of C.B-17 allotypes was monitored weekly. The T4 line showed 63% specific lysis at an effector:target ratio of 20:1 on <sup>51</sup>Cr-labeled CB101 cells at this time.

T10.1B.2C, and T10.1B.2D) show specificity patterns of lysis on different target cells similar to those observed with the primary Tc populations from which the clones were derived (Fig. 3). CB101 cells (Igh-1<sup>b</sup>) were lysed, but the nonproducing variant CB101NP, the U10 (Igh-1<sup>a</sup>), S10P (Igh-4<sup>b</sup>) cells, and mitogen-induced BALB/c blasts were not. Such a lysis pattern and the fact that affinity-purified Igh-1<sup>b</sup> protein was used as the priming immunogen (6) together suggest that the specificity of these Tc clones is directed to determinants of the Ig-1<sup>b</sup> Ig molecule.

Perhaps the strongest evidence for the Ig allotypic specificity of these Tc clones comes from the observation that purified Igh-1<sup>b</sup> will specifically inhibit the cytotoxic activity of Tc (Table II). Purified Igh-1<sup>a</sup>, Igh-4<sup>b</sup>, and Igh-4<sup>a</sup> failed to inhibit the activity of anti-Igh-1<sup>b</sup> Tc.

The results of our experiments on the question of MHC restriction must be regarded as preliminary. Two of the three clones display an unambiguous preference for Igh-1<sup>b</sup> targets of the self, *H-2<sup>d</sup>*, haplotype. The third shows the ability to cause extensive lysis of Igh-1<sup>b</sup>-expressing targets of the *H-2<sup>b</sup>* haplotype as well. At this point, however, it would be premature to conclude that this clone shows an unrestricted specificity for Igh-1<sup>b</sup>; alternatively, it may be partially cross-reactive on the *H-2<sup>b</sup>* haplotype. A more extensive sampling of Igh-1<sup>b</sup> targets with a variety of different MHC haplotypes would help to resolve this question, but these are currently unavailable.

*Mechanism of Inhibition by Soluble Igh-1<sup>b</sup>.* The finding that soluble Ig can inhibit lysis of Igh-1<sup>b</sup> targets has several interesting aspects that must be taken into account when considering the possible mechanisms involved. First, preincubation of the T cells with Ig is required; soluble Igh-1<sup>b</sup> does not inhibit cytotoxic activity when added during the assay. This is consistent with findings in other cytotoxic systems (2, 11). Second, incubation at 37°C is required (Table IV). Third, there is no demonstrable specific binding of Igh-1<sup>b</sup> (Table III).

The failure to show specific binding of Igh-1<sup>b</sup> proteins to these Tc clones may indicate that receptors are shed into the medium or that they are so few in number on the cell surface that they are not detectable by binding studies. Once Igh-1<sup>b</sup> binds to T cells by whatever means, e.g., via Fc receptors or by nonspecific adsorption, the cells then become lysable by surrounding Tc, resulting in allotype-specific autolysis (Table V). Such a possibility suggests that these allotype-specific Tc should be able to lyse



any neutral third party target cell, provided it has first been coated with Igh-1<sup>b</sup> proteins. However, preliminary attempts to couple Igh-1<sup>b</sup> to neutral target cells, using chromic chloride or via F<sub>c</sub> receptor binding, have not yielded positive results.

*Suppression of Igh-1<sup>b</sup> Expression In Vivo with Allotype-specific Killer Cells.* Aside from questions of the proximal target and the specificity of suppressor T cell activity, it remains to be determined whether a lytic mechanism is involved in allotype suppression. To some extent this possibility is supported by the finding (Table VII) that a clone of allotype-specific T cells, lytic for Igh-1<sup>b</sup>-expressing targets in vitro, is also able to inhibit expression of Igh-1<sup>b</sup> in vivo. In our view, these data raise the possibility that some aspects of allotype suppression and thus, of Ig regulation in general, may be mediated in vivo by killer T cells. However, it would not be prudent to consider that all suppressor T cells function via a lytic mechanism (12). Moreover, the demonstration in this paper that a T cell line exerts suppressor T cell activity in vivo need not necessarily imply that lysis and suppression are manifestations of the same function detected with different assays; alternatively, they could represent different functions, involving different mechanisms, of the same cell.

The above conclusion, that suppressor T cells can induce and/or maintain allotype suppression, supports the conclusion drawn in a previous paper (13) that allotype-bearing B cells are a target of T cells in allotype-suppressed mice.

### Summary

This study describes long-term-cultured lines and clones of cytotoxic T cells (T<sub>c</sub>) with specificity for determinants of the Igh-1<sup>b</sup> immunoglobulin allotype. These T<sub>c</sub> clones were initiated by repeated stimulation of immune spleen cells from BALB/c mice with an Igh-1<sup>b</sup>-producing myeloma, and then they were maintained in medium supplemented with mitogen-induced growth factors in the absence of further antigenic stimulation. The lytic potency of these clones was 30–100-fold greater than the primary cultures from which they were derived, and specificity studies showed them to be lytic for Igh-1<sup>b</sup> targets and not for targets expressing Igh-1<sup>a</sup> or Igh-4<sup>b</sup>, nor the lipopolysaccharide blasts. Finally, soluble preparations of Ig were tested for their ability to block lysis of labeled Igh-1<sup>b</sup>-expressing targets. The results showed that Igh-1<sup>b</sup> and not other immunoglobulin allotypes or isotypes could block lysis, and that the mechanism of lytic inhibition is due to Igh-1<sup>b</sup>-induced autolysis of the killer cells.

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