CHARACTERIZATION OF ANTIGEN-SPECIFIC, Ia-RESTRICTED, L3T4$^+$ CYTOLYTIC T LYMPHOCYTES AND ASSESSMENT OF THYMIC INFLUENCE ON THEIR SELF SPECIFICITY

By HANA GOLDING, TERRY I. MUNITZ, and ALFRED SINGER

From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

The specificity of many T cells for self major histocompatibility complex (MHC)[1]-encoded determinants is influenced by the MHC phenotype of the thymus in which their precursors had differentiated (1, 2). However, the intrathymic events that influence the developing T cell repertoire are poorly understood. To enhance our understanding of these events it is necessary to know if the thymus influences the self specificity of all T cells equally, or primarily influences the self specificity of certain subsets of T cells. Previous studies examining the self specificity of individual T cell subpopulations noted that, in both radiation bone marrow chimeras and thymic chimeras, the self specificity of class II–restricted T helper (Th) cells was profoundly skewed toward the haplotype of the chimeric thymus (3–5), whereas the self specificity of class I–restricted cytolytic T lymphocyte precursors (pCTL) did not appear to be significantly skewed toward the thymic haplotype (6–8). This dichotomy was not due to differences in the antigen responses used to assess the self specificities of these two T cell subpopulations, since the dichotomy was subsequently observed between antihapten Th cells and antihapten pCTL present in a single-response system to hapten-modified self (9, 10). Thus, these results indicated that, in the chimera model of T cell differentiation, not all T cell subpopulations were equally influenced by the MHC phenotype of the chimeric thymus. This concept has been further supported by the recent report (11) that CTL responses regulated by class II–encoded immune response (*Ir*) genes are determined by the *Ir* phenotype of the thymus, whereas CTL responses regulated by class I–encoded *Ir* genes are unaffected by the *Ir* phenotype of the chimeric thymus. However, since class II– and class I–restricted T cells differ in a variety of parameters, it is not clear which parameters are critical for distinguishing between T cell subpopulations whose self specificities are or are not profoundly influenced by the chimeric thymus. Among the parameters that could be critical are: (*a*) MHC class restriction specificity (class II vs. class I), (*b*) Lyt phenotype (L3T4 vs. Lyt-2), and (*c*) cellular function (help vs. kill).

[1] *Abbreviations used in this paper:* Con A, concanavalin A; Con A SN, concanavalin A–induced supernatant; CTL, cytolytic T lymphocytes; H, histocompatibility; *Ir*, immune response; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; pCTL, precursor cytolytic T lymphocyte; Th, T helper; TNP, trinitrophenyl.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/09/0943/19 $1.00 943
Volume 162 September 1985 943–961

TABLE I
MHC Alleles of Mouse Strains Used in this Study

Strain	H-2 alleles			
	K	I-A	I-E	D
C57BL/10 (B10)	b	b	—	b
B10.A	k	k	k	d
B10.A(4R)	k	k	—	b
B10.AQR	q	k	k	d
B10.BR	k	k	k	k
B10.D2	d	d	d	d
B10.GD	d	d	—	b
B10.Q	q	q	—	q
B10.T(6R)	q	q	—	d
BALB/c	d	d	d	d
C3H	k	k	k	k
C3H.SW	b	b	—	b

The present study was undertaken to identify some of the critical variables that can distinguish T cell subpopulations whose self specificities are profoundly influenced by the thymic haplotype from those that are not. In particular, we wished to assess the influence of the thymic haplotype on the self specificity of pCTL whose Lyt phenotype and MHC class restriction specificity were different from those of conventional Lyt-2$^+$ pCTL, but were identical to those of conventional L3T4$^+$ Th cells. Recently,[2] we found that primary class II allospecific pCTL can be activated in cultures containing supernatants rich in helper factors, and that they are of two distinct Lyt phenotypes, L3T4$^+$ Lyt-2$^-$ and L3T4$^-$ Lyt-2$^+$. In the present study we report the generation and relative precursor frequency of CTL that are antigen specific, self class II restricted, and L3T4$^+$. Using radiation bone marrow chimeras we also assess the influence of the chimeric thymus on the self-Ia specificity expressed by these class II–restricted, L3T4$^+$ CTL. Surprisingly, the results suggest that, in contrast to the influence of the thymus on L3T4$^+$, class II–restricted Th cells, the thymus does not significantly influence the self specificity of peripheral L3T4$^+$, class II–restricted pCTL.

Materials and Methods

Animals. The MHC alleles of the mouse strains used in this study are shown in Table I. Mice were purchased from The Jackson Laboratory, Bar Harbor, ME or were bred in our own animal colony.

Radiation Bone Marrow Chimeras. Radiation bone marrow chimeras are designated as bone marrow donor → irradiated recipient. An extensive description of the production and typing of such chimeras has been reported elsewhere (4). Briefly, recipient mice were irradiated with 950 rad from a ^{137}Cs source and were reconstituted 2–6 h later with 1.5×10^7 bone marrow cells that had been depleted of T cells by pretreatment with a rabbit anti-mouse brain serum, a reagent specifically cytotoxic for all T cells (12), plus guinea pig complement. Spleen cells were obtained from each chimera no earlier than 2 mo after irradiation and bone marrow reconstitution, at which time they were >98% of donor

[2] Golding, H., and A. Singer. 1985. Specificity, phenotype, and precursor frequency of primary cytolytic T lymphocytes specific for class II major histocompatibility antigens. *J. Immunol.* In press.

bone marrow origin as assessed by indirect immunofluorescence, and were tolerant to donor and host MHC determinants as assessed by cell-mediated lympholysis and mixed lymphocyte proliferation.

Monoclonal Antibodies (mAb). Monoclonal anti-I-$A^{b,d}$ antibody was protein A–purified mouse ascites of the hybridoma cell line 25-9-17, and was used at a final concentration of 25 μg/ml (13). Monoclonal anti-I-A^k antibody was the culture supernatant of the 10-2.16 hybridoma and was used at a final concentration of 25% (14). Monoclonal anti-Lyt-2.2 antibody was a culture supernatant of the hybridoma cell line 83-12-5 (IgMκ) produced by Dr. J. Bluestone, National Institutes of Health, Bethesda, MD. Monoclonal anti-L3T4 antibody was a culture supernatant of either the hybridoma cell line GK1.5, provided by Dr. Frank Fitch, Chicago, IL (15) or the hybridoma cell line H129.1a provided by Dr. Michael Pierres, Marseilles, France (16), as indicated.

Concanavalin A Supernatant (Con A SN). Con A SN was the 18-h SN from Con A–stimulated BALB/c spleen cells, as described (17). The Con A SN was supplemented with 0.2 M α-methyl-D-mannoside to neutralize the remaining Con A and was used in culture at a final concentration of 25%.

In Vivo Priming to Minor Histocompatibility (H) Antigens. Where indicated, mice were primed intraperitoneally with 2×10^7 minor H disparate spleen cells, at least 2 wk before use.

In Vitro Generation of CTL. 2-ml mixed lymphocyte cultures consisted of 4×10^6 responder cells and 4×10^6 stimulator cells (2,000-rad–irradiated whole spleen cells) (9). Trinitrophenyl (TNP) modification of stimulator cells was performed with 10 mM trinitrobenzene sulfonate as previously described (18). Cells were cultured in a 7.5% humidified air atmosphere in RPMI 1640 plus 10% fetal calf serum supplemented, where indicated, with 25% Con A SN. On day 5, cultures were assayed for CTL generation by their ability to lyse ^{51}Cr-labeled target cells in a 4 h assay. Percent specific lysis was calculated as: $100 \times$ [(experimental − spontaneous release)/(maximum − spontaneous release)]. CTL were assayed over four effector/target (E/T) ratios, and each experimental point represents the mean of at least three replicate cultures. For simplicity, only one or two E/T ratios are shown. Standard errors were always <5% of the mean.

Target Cells. Heterogeneous target cell populations were either 2-d lipopolysaccharide (LPS)- or Con A–induced spleen cell blasts, as indicated. Monoclonal target cell populations were either M12.4.1 or M12.C3 cell lines, generously provided by Dr. Laurie Glimcher, Harvard Medical School, Boston, MA. M12.4.1 is an H-2^d, Ia^+ B lymphoblastoid cell (19); M12.C3 is an Ia^- variant cell line derived from M12.4.1 by gamma irradiation and negative immunoselection.[3] The M12.C3 cell line continues to express H-$2K^d$, D^d, and L^d but does not express detectable amounts of either I-A- or I-E-encoded determinants on its cell surface.[3] TNP modification of each target cell population was performed with 10 mM trinitrobenzene sulfonate as described (18).

Depletion of Lyt-2^+ Responder Cells. Responder cells were incubated with a 1:50 dilution of culture supernatant from 83-12-5 hybridoma (anti-Lyt-2.2) cell line at 10^6 cells/ml for 30 min on ice. Cells were then pelleted, resuspended in complement (C), and incubated for 45 min at 37°C. Cells were then washed thoroughly before addition to culture.

Limiting Dilution Analysis. Limiting dilution analysis for determination of pCTL frequencies was performed in U-bottomed microtiter plates. Graded numbers of responding spleen cells were cultured (at least 24 replicates for each responder cell number) with either no or 10^6 irradiated (2,000 rad) stimulator cells per microculture. Each microculture contained a total volume of 0.2 ml RPMI 1640 plus 10% fetal calf serum supplemented with Con A SN (25% final concentration). The microcultures were incubated for 7 d in a 7.5% CO_2 atmosphere. On day 7, 1,500 ^{51}Cr-labeled, TNP-modified or -unmodified LPS targets were added to each well and the percent ^{51}Cr release was determined at the end of 4 h. Positive cultures were defined as those with ^{51}Cr release values exceeding the mean spontaneous release by ≥3 SD. The minimal estimates of pCTL frequencies were

[3] Glimcher, L. H., D. J. McKean, E. Choi, and J. G. Seidman. 1985. Complex regulation of class II gene expression: analysis with class II mutant cell lines. Submitted for publication.

determined by Poisson statistics (20). The coefficient of correlation in each experiment was >0.97.

Results

Generation of TNP-specific, Self-Ia–restricted, L3T4$^+$ Primary CTL. As an initial attempt to generate class II–restricted, anti-TNP CTL, we cultured Lyt-2-depleted as well as unfractionated responder spleen cells with irradiated stimulator cells in the presence of Con A SN and then assayed them on LPS-induced spleen cell blasts as target cells. As can be seen in Table II, unprimed and unfractionated B10.T(6R) spleen cells in the presence of Con A SN generated some CTL specific for class I and class II MHC alloantigens (groups 1 and 3) and others specific for TNP-modified self antigens (group 5). After anti-Lyt-2 plus C treatment of the 6R responding cells, no CTL were elicited in response to class I disparate B10.Q stimulator cells (group 2), confirming that class I–allospecific pCTL are Lyt-2$^+$ and indicating that the responding cell population was effectively depleted of Lyt-2$^+$ T cells. However, CTL were generated from the same Lyt-2$^-$ responders upon stimulation with class II disparate B10.AQR stimulator cells (group 4), consistent with our earlier finding[2] that there exists a subset of primary class II–allospecific pCTL that are L3T4$^+$ Lyt-2$^-$. More importantly, CTL were also generated from the same Lyt-2$^-$ 6R responders upon stimulation with TNP-self (group 6), albeit at a reduced level relative to unfractionated responders (group 5). It should be noted that these CTL effectors were TNP specific in that they lysed only TNP-modified LPS targets (groups 5 and 6), and their generation from Lyt-2$^-$ responder populations required the presence in culture of Con A SN (data not shown).

Since conventional CTL are restricted to class I MHC determinants and arise from Lyt-2$^+$ precursors (21), it seemed a reasonable possibility that the anti-TNP CTL generated from Lyt-2$^-$ responder cells were class II rather than class I restricted. This possibility was first examined by comparing the lysis of TNP-modified Ia$^+$ and Ia$^-$ target cells (Table III). As can be seen in experiment 1, anti-TNP CTL generated from Lyt-2$^-$ responding cells lysed Ia$^+$, TNP-modified LPS blasts but did not lyse Ia$^-$, TNP-modified Con A blasts. In contrast, anti-TNP CTL generated from unfractionated responding cells lysed both targets equally well (Table III, Exp. 1). In a second experiment (Table III, Exp. 2), the Ia$^+$ B lymphoblastoid cell line M12.4.1 and an Ia$^-$ variant of this cell line, M12.C3, both of which express H-2^d class I determinants, were used as target cells.[3] Again, anti-TNP CTL generated from Lyt-2$^-$ responders, unlike anti-TNP CTL from unfractionated responders, lysed the Ia$^+$, TNP-modified target cells but failed to lyse the Ia$^-$, TNP-modified target cells (Table III, Exp. 2).

To ascertain whether anti-TNP CTL generated from Lyt-2$^-$ responders actually used Ia determinants as their MHC restriction specificity, we tried to block their lysis of TNP-modified target cells with Ia-specific mAb (Table IV). In experiment 1, Lyt-2$^-$ (B10 × B10.BR)F_1 responders were stimulated with TNP-modified cells of either parental haplotype. Upon stimulation with either TNP-B10 or TNP-B10.BR stimulator cells, CTL were generated that lysed TNP-B10 × B10.A(4R) target cells that express both I-A^b and I-A^k (but no I-E) parental determinants (Table IV). Lysis of these target cells by Lyt-2$^-$ F_1 CTL that were

TABLE II

Unprimed Lyt-2⁻ Spleen Cells Do Not Contain pCTL Specific for Class I MHC Antigens But Do Contain pCTL Specific for Class II and TNP-Self Antigens

Group	Responder		Stimulator	Stimulator cell antigen	Con A* SN		Percent specific lysis on LPS blasts						
	Strain	Treatment					B10.Q		B10.AQR		TNP-6R		B10.T(6R)
						E/T:	80	40	80	40	80	40	80
1	B10.T(6R)	C′	B10.Q	D^q	+		41	32					−4
2		Anti-Lyt-2 + C′			+		−7	−3					−2
3		C′	B10.AQR	I^k	+				60	50			5
4		Anti-Lyt-2 + C′			+				58	44			1
5		C′	TNP-B10.T(6R)	TNP-Self	+						65	56	−3
6		Anti-Lyt-2 + C′			+						35	32	−2

* Con A SN was added at 25% final concentration.

TABLE III

Lyt-2$^-$ Anti-TNP CTL Lyse Ia$^+$ But Not Ia$^-$ Targets

Exp.	Responder		Stimulator	Con A SN*	Percent specific lysis of:				
					TNP-B10.T(6R)				B10.T(6R)
	Strain	Treatment			LPS blasts		Con A blasts		LPS blasts
				E/T:	80	40	80	40	80
1	B10.T(6R)	C′	TNP-B10.T(6R)	+	77	61	70	53	2
		Anti-Lyt-2 + C′		+	30	14	2	0	1
					TNP-M12.4.1‡ (Ia$^+$)		TNP-M12.C3§ (Ia$^-$)		M12.4.1 (Ia$^+$)
					80	40	80	40	80
2	B10.GD	C′	TNP-B10.GD	+	46	44	48	31	4
		Anti-Lyt-2 + C′		+	30	23	0	0	−7

* Con A SN was added at 25% final concentration.

‡ M12.4.1 is an Ia$^+$ B lymphoma line of BALB/c origin.

§ M12.C3 is an irradiation-induced mutant of M12.4.1 that expresses class I but not class II MHC molecules on its cell surface.

generated in response to TNP-B10.BR stimulators was completely inhibited by anti-I-A^k mAb (Table IV, group 4), whereas lysis of the same target cells by Lyt-2$^-$ F_1 CTL that were generated in response to TNP-B10 stimulators was unaffected by anti-I-A^k mAb (group 3). These results demonstrate that (*a*) Lyt-2$^-$ anti-TNP F_1 CTL distinguish between parental Ia determinants, and (*b*) blocking of target cell lysis by anti-I-A^k mAb is specific since it is entirely dependent upon the specificity of the CTL effectors. Consequently, this experiment demonstrates that Lyt-2$^-$ anti-TNP CTL are restricted by polymorphic Ia determinants and that the self-Ia polymorphisms which they recognized are those expressed by the TNP stimulator cells against which they were generated.

Finally, to demonstrate that the CTL effectors generated from Lyt-2$^-$ responder cells were L3T4$^+$ as well as Ia-restricted, we tried to block their lysis of target cells by anti-L3T4 and anti-I-A mAb. In Table IV, experiment 2, anti-TNP CTL were generated from B10.AQR spleen cells and assayed on TNP-modified B10.A(4R) target cells that express only I-A^k class II determinants. It can be seen that both anti-I-A^k and anti-L3T4 mAb inhibited the lysis of TNP-4R targets by anti-TNP CTL generated from Lyt-2$^-$ responders (Table IV, group 8), but did not inhibit lysis of the same target cells by either anti-TNP CTL or anti-K^k CTL generated from unfractionated responders (which would be expected to mainly be Lyt-2$^+$, class I–restricted CTL) (groups 5 and 6). Taken together, these data demonstrate that Lyt-2$^-$, unprimed T cells can be triggered by antigen and Con A SN to generate L3T4$^+$ CTL effectors that are TNP specific and self-Ia restricted.

Class II–restricted, Anti-TNP Primary CTL Do Not Lyse Bystander Target Cells. Because their Lyt phenotype and MHC restriction specificity are identical to that of anti-TNP Th cells (10, 22), it was conceivable that the TNP-specific and Ia-restricted CTL generated in primary cultures were helper cells that nonspecifically lysed target cells by secreting lymphotoxins. Precisely such a lytic mechanism, capable of killing bystander target cells, has recently been ascribed to several antigen-specific Th clones (23). Consequently, we examined the ability

TABLE IV

Lysis of TNP-modified LPS Blasts by Lyt-2⁻ Effector Cells Is Blocked by Anti-Ia and Anti-L3T4 Monoclonal Antibodies

Exp.	Group	Responder		Stimulator	Stimulator cell antigen	Con A SN	Target:	Percent specific lysis of LPS blast* in the presence of mAb			
								TNP-B10 × B10.A(4R)			
							mAb:‡	No Ab		Anti-I-A^k	
		Strain	Treatment				E/T:	80:1	40:1	80:1	40:1
1	1	B10 × B10.BR	C′	TNP-B10	TNP-H-2^b	+		82	72	86	71
	2	B10 × B10.BR		TNP-B10.BR	TNP-H-2^k	+		90	82	92	80
	3	B10 × B10.BR	Anti-Lyt-2 + C′	TNP-B10	TNP-H-2^b	+		27	35	32	21
	4	B10 × B10.BR		TNP-B10.BR	TNP-H-2^k	+		30	27	3	2
								TNP-B10.A(4R)					
								No Ab		Anti-I-A^k		Anti-L3T4	
								80:1	40:1	80:1	40:1	80:1	40:1
2	5	B10.AQR	C′	B10.A	K^k	+		52	46	44	42	51	55
	6	B10.AQR		TNP-B10.AQR	TNP-Self	+		65	62	65	55	67	58
	7	B10.AQR	Anti-Lyt-2 + C′	B10.A	K^k	+		2	0	ND§		ND	
	8	B10.AQR		TNP-B10.AQR	TNP-Self	+		25	18	3	3	5	2

* Lysis of unmodified targets was ≤2% in all groups other than 5 and 7.

‡ Blocking mAb used: 10-2-16 (anti-I-A^k) culture SN (25% vol/vol). H129.1a, rat mAb (anti-L3T4) culture SN was used at 25% (vol/vol) final concentration.

§ ND, not done.

of L3T4+ anti-TNP primary CTL to lyse bystander target cells. Anti-TNP CTL generated from Lyt-2− B10.T(6R) responder populations lysed TNP-modified 6R targets but did not lyse inappropriate B10.Q targets that express D^q alloantigens (Table V, group 1). More importantly, these CTL effectors did not lyse inappropriate B10.Q targets even in the presence of appropriate TNP-6R target cells. The B10.Q targets were lyseable, as shown by the fact that they were killed by anti-D^q CTL effectors (Table V, group 2). This experiment suggests that primary class II–restricted, anti-TNP CTL, like conventional CTL effectors, lyse their target cells by a mechanism involving a direct effector cell–target cell interaction.

L3T4+, Class II–restricted CTL Also Recognize Minor Histocompatibility (H) Antigens. To rule out the possibility that TNP was unique in being recognized by L3T4+, class II–restricted CTL, we next attempted to generate L3T4+, class II–restricted CTL specific for minor H antigens. Spleen cells from mice previously primed in vivo with minor H disparate spleen cells (24) were restimulated in vitro with minor H disparate stimulator cells in the presence of Con A SN. In Table VI, Exp. 1, CTL effectors that were generated from Lyt-2− B10 responder cells lysed C3H.SW target cells but lysed neither B10 nor C3H target cells, indicating that they were minor H specific and MHC restricted. More importantly, their lysis of C3H.SW target cells was significantly inhibited by anti-I-A^b mAb, indicating that these anti–minor H CTL were self-Ia restricted. Blocking by this anti-I-A^b mAb was specific, since this same mAb did not interfere with lysis of the same C3H.SW target cells by conventional class I–restricted, minor H–specific CTL effectors generated from unfractionated responder cells. In Table VI, Exp. 2, anti–minor H CTL were again generated from a primed Lyt-2− responding cell population. In this experiment, the resulting CTL effectors were directly phenotyped as L3T4+ since their lysis of appropriate minor H–bearing target cells was significantly inhibited by anti-L3T4 mAb. We conclude that self-Ia-restricted, L3T4+, pCTL have a receptor repertoire for nominal antigen that at least includes TNP and minor H antigens.

Influence of the Thymus on the Self-Ia Specificity of L3T4+ pCTL. Since we had previously observed (9, 10, 22) in chimeric animals that the self-Ia specificity of peripheral L3T4+ anti-TNP Th cells was skewed dramatically toward the thymic haplotype but that the self specificity of peripheral anti-TNP pCTL was not, we were especially interested to determine if the self-Ia specificity of peripheral

TABLE V

Lyt-2− Anti-TNP CTL Do Not Lyse Bystander Target Cells

Group	Responder		Stimulator	Con A SN	Percent specific lysis of ^{51}Cr-labeled LPS blasts in the presence of unlabeled LPS blasts:				
	Strain	Treatment			*B10.T(6R) −	*TNP-6R −	*B10.Q −	*B10.Q + TNP-6R	*B10.T(6R) + TNP-6R
1	B10.T(6R)	Anti-Lyt-2 + C′	TNP-B10.T(6R)	+	1	30	4	6	2
2		C′	B10.Q	+	8	34	90		
3		Anti-Lyt-2 + C′	B10.Q	+	0	0	8		

* Effectors were mixed with equal numbers of ^{51}Cr-labeled (*) and unlabeled targets at an E/T ratio of 80:1 effector cells to ^{51}Cr-labeled LPS blasts.

TABLE VI

Generation of L3T4$^+$ CTL that Recognize Minor H Antigens in the Context of Self-Ia Determinants

Exp.	Responder*			Stimulator	Stimulator cell antigen	Con A SN	E/T ratio	Percent specific lysis of LPS blasts in the presence of mAb:‡			
	Strain	In vivo priming	In vitro treatment					C3H.SW		B10	C3H
								No mAb	Anti-I-A^b	No mAb	No mAb
1	B10 (H-2^b)	C3H.SW (H-2^b)	C′	C3H.SW	H-2^b + C3H minor H	+	40:1	69	79	14	6
							10:1	42	43	−9	−1
			Anti-Lyt-2 + C′			+	40:1	42	22	7	6
							10:1	20	7	0	−7
								BALB/c		B10.D2	
								No mAb	Anti-L3T4	No mAb	
2	B10.D2 (H-2^d)	BALB/c (H-2^d)	C′	BALB/c	H-2^d + BALB minor H	+	40:1	91	94	4	
							10:1	88	80	2	
			Anti-Lyt-2 + C′			+	40:1	45	12	3	
							10:1	24	3	3	

* Responder mice were primed with 20×10^6 spleen cells intraperitoneally at least 3 wk before secondary stimulation in vitro. Cultures contained 4×10^6 primed responder cells and 4×10^6 2,000-rad–irradiated stimulator cells.

‡ Blocking mAb: 25-9-17 (anti-Ia^b) protein A–purified mAb was used at 25 μg/ml final concentration. H129.1a rat mAb (anti-L3T4) culture SN was used at 25% (vol/vol).

L3T4$^+$ anti-TNP pCTL was skewed toward the haplotype of the chimeric thymus. To address this question, we used responder spleen cells from both A → B and F_1 → parent radiation bone marrow chimeras for anti-TNP CTL responses.

First, we verified the influence of the chimeric thymus on the self specificity of the anti-TNP Th cells from the A → B and F_1 → parent chimeras used in this study (Table VII). Chimeric spleen cells were stimulated with TNP-modified parental cells and assayed on TNP-modified LPS blasts as target cells. In fully allogeneic A → B chimeras, the influence of the chimeric thymus on anti-TNP Th cells was easily demonstrated since, in the absence of Con A SN, the chimeric spleen cells were only stimulated by host type, and not donor type, parental stimulator cells (Table VII, Exp. 1). As previously observed (9), differences in response of pCTL to the two parental stimulator cell populations was not observed when the requirement for Th cell activation was circumvented by the addition to culture of Con A SN (Table VII, Exp. 1). In F_1 → parent chimeras it was somewhat more difficult to assess the influence of the chimeric thymus on anti-TNP Th cells since it was necessary to deplete the responding chimeric cells of F_1 accessory cells so that all the accessory cells in the response cultures would be of parental stimulator type. Indeed, unfractionated F_1 → parent responder cells responded equally well to stimulator cells of either parental haplotype: however, F_1 → parent responder cells that were depleted of endogeneous F_1 accessory cells by passage over Sephadex G-10 (25) generated anti-TNP CTL only in response to host type parental stimulator cells (Table VII, Exp. 2), and this restriction could be circumvented by supplementing the G-10-passed responders with Con A SN. These results verify that, in the experimental animals

TABLE VII

Self Specificity of Anti-TNP Th Cells But Not of Anti-TNP CTL Is Skewed Toward the Haplotype of the Chimeric Thymus

Exp.	Responder		Con A SN*	Stimulator:	Percent specific lysis of LPS blasts‡	
	Strain	Treatment		Target:	TNP-B10 TNP-B10	TNP-B10.BR TNP-B10.BR
1	B10 → B10.BR	–	–		0	37
		–	+		61	50
	B10.BR → B10	–	–		24	1
		–	+		51	31
	B10 × B10.BR	–	–		29	31
		–	+		58	57
2	B10 × B10.BR → B10	–	–		39	38
		G10	–		34	1
		G10	+		73	72
	B10 × B10.BR → B10.BR	–	–		23	35
		G10	–		0	29
		G10	+		62	58
	B10 × B10.BR	–	–		38	44
		G10	–		27	27
		G10	+		72	62

* Con A SN was added where indicated at 25% final concentration.

‡ All values represent percent specific lysis of targets at 80:1 E/T ratios. In all groups, killing of unmodified targets was ≤2%.

used in the present study, the self specificity of anti-TNP Th cells was significantly skewed toward the haplotype of the chimeric thymus, whereas the self specificity of anti-TNP CTL was not.

To determine whether class II–restricted, L3T4+, anti-TNP primary CTL could be generated from these chimeras, unfractionated and Lyt-2− spleen cells from F_1 → parent chimeras were cultured with TNP-modified parental stimulators in the presence of Con A SN (Table VIII). Contrary to our expectation, Lyt-2− responder cells from F_1 → parent chimeras were stimulated equally well by TNP-modified cells of either parental type. The anti-TNP CTL generated from chimeric Lyt-2− responder cells were documented to be L3T4+ since their lysis of TNP-F_1 target cells was significantly blocked by anti-L3T4 mAb (Table VIII). These results suggested that, unlike the self specificity of L3T4+ anti-TNP Th cells in F_1 → parent chimeras, the self specificity of L3T4+ anti-TNP pCTL in these same mice was not skewed toward the thymic haplotype.

The same question was next approached with A → B allogeneic chimeras. In Figs. 1 and 2, anti-TNP CTL effectors were assayed on TNP-[B10 × B10.A(4R)] target cells that express both I-A^b and I-A^k but not I-E determinants. As was observed with F_1 → parent chimeras, Lyt-2− responder cells from B10 → B10.BR and B10.BR → B10 fully allogeneic chimeras were comparably stimulated by

TABLE VIII

Self-Ia Specificity of L3T4$^+$ Lyt-2$^-$ Anti-TNP CTL Is Not Skewed Toward the Haplotype of the Chimeric Thymus

Responder		Stimulator	Con A SN	mAb	Percent specific lysis of LPS blasts in the presence of mAb:*				
					TNP-(B10 × B10.BR)F$_1$				F$_1$
				E/T	None		Anti-L3T4		None
Strain	Treatment				80:1	40:1	80:1	40:1	80:1
F$_1$ → B10.BR	Anti-Lyt-2 + C′	TNP-B10	+		20	17	5	4	−4
		TNP-B10.BR	+		24	19	4	3	−5
F$_1$ → B10	Anti-Lyt-2 + C′	TNP-B10	+		34	22	12	5	8
		TNP-B10.BR	+		24	18	3	0	0
(B10 × B10.BR)F$_1$	Anti-Lyt-2 + C′	TNP-B10	+		24	13	6	3	−6
		TNP-B10.BR	+		27	13	3	0	−3
(B10 × B10.BR)F$_1$	C′	TNP-B10	+		54	51	64	53	−4
		TNP-B10.BR	+		57	53	61	55	−5

* Rat mAb GK1.5 (anti-L3T4) culture SN was added to CTL effectors at 25% (vol/vol) final concentration.

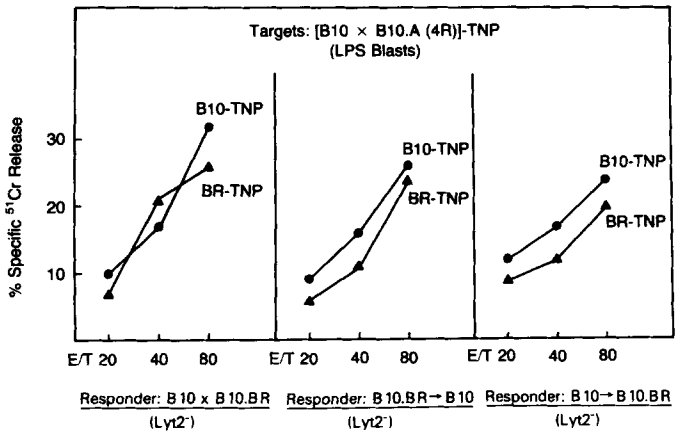

FIGURE 1. In allogeneic bone marrow chimeras, the repertoire of Lyt-2$^-$ anti-TNP CTL is not skewed toward the thymic I-A haplotype. Anti-Lyt-2.2 + C′–treated spleen cells from B10 × B10.BR (*left*), B10.BR → B10 chimera (*middle*), or B10 → B10.BR chimera (*right*) were stimulated in culture with 2,000-rad–irradiated TNP-B10 (●) or TNP-B10.BR (▲) stimulator cells. At the end of 5 d, effectors were tested for their ability to lyse [B10 × B10.A(4R)]F$_1$-TNP LPS targets in a 4-h ^{51}Cr-release assay at the indicated E/T ratios. Lysis of unhaptenated F$_1$ targets or syngeneic targets was ≤2%.

TNP stimulators of either parental haplotype (Fig. 1). As possible explanations for this result, we considered that (*a*) the self-Ia specificity of Lyt-2$^-$ anti-TNP chimeric pCTL was in fact skewed by the chimeric thymus but that these anti-TNP pCTL could not distinguish TNP-I-A^k from TNP-I-A^b determinants, or (*b*) the chimeric CTL generated in response to TNP-modified donor stimulators were not in fact Ia restricted, even though they were L3T4$^+$. We tried to test these alternatives by blocking the lysis of TNP-B10 × 4R target cells by Lyt-2$^-$

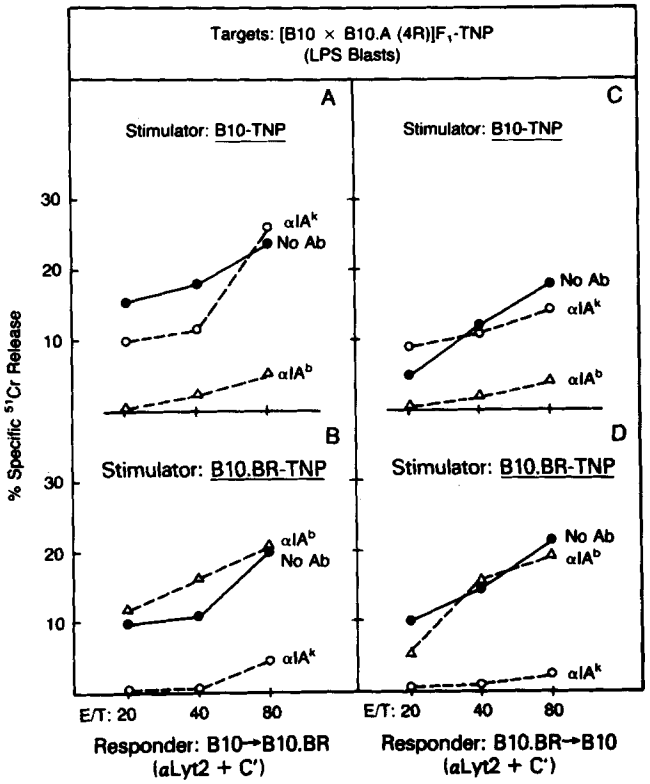

FIGURE 2. Anti-Ia mAb blocking of Lyt-2⁻ anti-TNP CTL from allogeneic bone marrow chimeras. Anti-Lyt-2.2 + C′-treated spleen cells from B10 → B10.BR chimera (*A*, *B*) or B10.BR → B10 chimera (*C*, *D*) were stimulated in culture with 2,000-rad-irradiated B10-TNP (*A*, *C*) or B10.BR-TNP (*B*, *D*) stimulator cells. At the end of 5 d, effectors were tested for their ability to lyse [B10 × B10.A(4R)]F_1-TNP ^{51}Cr-LPS targets in the presence of: no Ab (●), anti-I-A^k mAb (10-2-16, 25% concentration) (○), or anti-I-A^b (25-9-17, 25 μg/ml) (△). Targets were preincubated with the mAb for 30 min before the addition of effectors at the indicated E/T ratios. Lysis of unhaptenated F_1 targets or syngeneic targets was <2%.

anti-TNP chimeric CTL with either I-A^b- or I-A^k- specific mAb (Fig. 2). Even though both mAb bound to the TNP-F_1 target cells, each effector population, whether generated in response to TNP-modified stimulators of donor or host haplotype, was significantly blocked by either the anti-I-A^b or the anti-I-A^k mAb, but not by both. Invariably, the particular anti-I-A mAb that blocked each chimeric effector population was the one specific for the Ia determinants expressed by the TNP stimulators against which the CTL were raised, and was not necessarily the mAb specific for the haplotype of the chimeric thymus from which the responder cells were derived (Fig. 2). It should be noted that the blocking observed by each mAb was specific, since each mAb served as the negative control for the other. These results clearly demonstrate that anti-TNP CTL generated from Lyt-2⁻ chimeric cells are restricted to either donor or host Ia determinants, depending not on the haplotype of the chimeric thymus but only on the haplotype of the TNP-modified stimulators against which they were raised. In addition, since each CTL effector population was significantly inhibited

TABLE IX

Frequency Analysis of Anti-TNP pCTL in Unfractionated and Lyt-2⁻ Responder Cell Populations

Responder	Stimulator	No. of CTL precursors per 10^6 spleen cells*	
		Responder cell pretreatment	
		C′	Anti-Lyt-2 + C′
(BALB/c × B6)F_1	B10-TNP	78	6.9
	B10.D2-TNP	62	6.7
B10 → B10.D2	B10-TNP	25	2.1
	B10.D2-TNP	38	3.3
B10.D2 → B10	B10-TNP	21	3.4
	B10.D2-TNP	10	1.5

* The limiting dilution analysis was conducted as described in Material and Methods. TNP-(BALB/c × B6)F_1 ^{51}Cr-labeled LPS blasts were used as targets. The correlation coefficients of the regression lines generated were all ≥0.97. Poisson statistics were used to calculate the precursor frequencies.

in its lysis of TNP-F_1 target cells by mAb directed against only one of the two parental I-A determinants expressed by the TNP-F_1 target cells, we conclude that each CTL effector population did distinguish between TNP-modified I-A^b and I-A^k parental determinants.

It was possible that the presence of Con A SN in bulk cultures might have obscured significant skewing of the self-Ia specificity expressed by Lyt-2⁻ anti-TNP CTL precursors by inducing the expansion of only minor pCTL subpopulations. Therefore, we next conducted limiting dilution experiments to examine this possibility. To permit comparisons, results are expressed as the number of total or Lyt-2⁻ anti-TNP CTL precursors per 10^6 spleen cells that could be stimulated to respond to either parental haplotype in normal F_1 mice and A → B fully allogeneic chimeras (Table IX). In both normal and chimeric animals the frequency of Lyt-2⁻ anti-TNP pCTL was ~10% of the total anti-TNP pCTL detected. More importantly, in chimeric mice, the number of anti-TNP pCTL responsive to TNP stimulators of host type from unfractionated or Lyt-2⁻ responder populations was only approximately twofold the number responsive to TNP stimulators of donor type. Parallel cultures assayed on unmodified target cells revealed no positive wells, indicating that essentially all lysis of TNP-modified target cells was due to TNP-specific pCTL. These findings demonstrate that the chimeric thymus only minimally influences the self specificity expressed by peripheral anti-TNP pCTL, even those that are L3T4⁺ Lyt-2⁻.

Discussion

The goals of this study were to generate antigen-specific L3T4⁺ CTL, determine their MHC restriction specificity, and assess the potential influence of the thymus on their self specificity. We found that (*a*) L3T4⁺ CTL could be readily generated from Lyt-2⁻ responder T cell populations upon stimulation with

antigen in the presence of Con A SN; (*b*) the antigens recognized by L3T4$^+$ CTL included TNP and minor H; (*c*) antigen recognition by L3T4$^+$ CTL was restricted by self-Ia determinants; and (*d*) in radiation bone marrow chimeras, the self-Ia specificity of L3T4$^+$ anti-TNP CTL, unlike that of L3T4$^+$ anti-TNP Th cells, was only marginally skewed toward the haplotype of the chimeric thymus. Thus, the present study suggests that the chimeric thymus does not markedly skew the self specificity of peripheral pCTL, regardless of their Lyt phenotype or MHC restriction specificity.

In contrast to L3T4$^+$ CTL specific for class II MHC alloantigens that have been described previously (16),[2] very little is known about L3T4$^+$ CTL specific for non-MHC antigens. Most antigen-specific CTL are Lyt-2$^+$, derive from Lyt-2$^+$ precursors, and use class I MHC determinants as restricting elements (21). By depleting Lyt-2$^+$ T cells from the responding T cell population, we avoided generation of Lyt-2$^+$ CTL and so were able to observe the generation of L3T4$^+$ CTL, provided that the cultures were supplemented with supernatants rich in helper factors, and that the CTL were assayed on target cells bearing Ia determinants. The CTL generated from Lyt-2$^-$ responder cells were not simply residual Lyt-2$^+$ cells that had clonally expanded in the presence of Con A SN since their lysis of appropriate target cells was blocked by anti-L3T4 mAb; this simultaneously phenotyped the CTL effectors as L3T4$^+$ and suggested that the L3T4 molecule was involved in their lysis of target cells. Since we observed that L3T4$^+$ self-Ia-restricted murine CTL recognized TNP and minor H nominal antigens as well as MHC alloantigens, it is conceivable that the specificity of their receptor repertoire is as broad as that of conventional Lyt-2$^+$ class I–restricted CTL, although restricted to class II rather than class I MHC determinants. However, the number of L3T4$^+$ anti-TNP pCTL in the spleens of normal mice, as determined by limiting dilution analysis, was only 5–10% that of L3T4$^-$, Lyt-2$^+$ anti-TNP pCTL. It should be noted that this number probably does not reflect the relative frequency of all anti-TNP pCTL that are class II restricted since it is possible that a subset of Lyt-2$^+$ anti-TNP pCTL may also be class II restricted. This possibility is based on an analogy to our earlier finding[2] for recognition of MHC alloantigens: that Lyt-2$^+$ primary pCTL recognize class I as well as class II MHC alloantigens, whereas L3T4$^+$ primary pCTL recognize only class II MHC alloantigens.

The existence of antigen-specific, class II–restricted, L3T4$^+$ CTL provided us with the possibility of making comparisons between functionally distinct but otherwise similar T cell subsets. We previously observed (9) that, in chimeric mice, the self specificities of anti-TNP Th cells and anti-TNP pCTL were not equally influenced by the haplotype of the chimeric thymus. We suspected that the critical difference might be the MHC class restriction expressed by these two different T cell subsets, but the possibility could not be excluded that differences other than MHC class restriction were actually the important ones. Consequently, we wished to assess the influence of the chimeric thymus on the self-Ia specificity expressed by anti-TNP T cells that were CTL but were otherwise similar to anti-TNP Th cells with regard to MHC class restriction and Lyt phenotype. The present report demonstrates, by bulk culture experiments and precursor frequency analysis, that the self-Ia specificity expressed by L3T4$^+$ anti-TNP pCTL

from radiation bone marrow chimeras is not significantly skewed toward the haplotype of the chimeric thymus. These results contrast markedly with those for self-Ia-restricted, $L3T4^+$, anti-TNP Th cells; together, they support the concept that functionally distinct T cell subsets have significantly different differentiation requirements. It should be noted that we examined $L3T4^+$ anti-TNP CTL rather than $L3T4^+$ anti–minor H CTL because the phenotype and specificity of anti–minor H Th cells remain controversial (26), preventing ready comparisons between the MHC restriction specificities and phenotypes of anti–minor H Th and pCTL.

The marked influence of the chimeric thymus on the self-Ia specificity of anti-TNP $L3T4^+$ Th but not pCTL suggests that, unlike Th cells, CTL do not have an obligatory intrathymic differentiation step, regardless of their MHC restriction specificity. We have previously suggested (7, 8) that peripheral class I–restricted CTL derive from two distinct precursor lineages, one that differentiated intrathymically and one, extrathymically. The present finding of a small but demonstrable twofold skewing toward the thymic haplotype is compatible with the existence of both intra- and extrathymic differentiation pathways for class II–restricted CTL as well. It is of interest to note that Th cells and CTL have recently been found (27) to differ in their use of the gene encoding the gamma chain of the T cell receptor, although examination of class II–restricted CTL for gamma chain expression has not yet been reported. An intriguing possibility is that differences in gamma chain expression of Th and CTL might result not from differences in their MHC restriction specificity but rather from differences in their differentiation history, in that an intrathymic differentiation step that appears to be obligatory only for Th cells might also be obligatory for switching off gamma chain expression.

However, it is possible to reconcile the present results with the existence of an obligatory intrathymic differentiation step for all class II–restricted T cells if functionally distinct T cells are either selected on different thymic elements or differentiate in different thymic compartments. For example, it is conceivable that Th cells are selected on Ia^+ elements whereas pCTL are primarily selected on Ia^- elements, so that the self specificity of Ia-restricted pCTL would not be influenced by the thymic Ia phenotype. Studies (28) with neonatal animals whose expression of Ia determinants was suppressed by chronic in vivo administration of anti-Ia mAb are quite consistent with this possibility. Alternatively, if Th cells and pCTL differentiate in different thymic compartments that are repopulated at different rates by Ia^+ donor bone marrow elements (29), Th cells might be exposed in the thymus to only host Ia elements, whereas pCTL might be exposed to both host and donor Ia elements. Recent evidence (30) from the avian system is consistent with the possibility that some thymic compartments are more rapidly repopulated by donor Ia^+ cells than others.

The most obvious concerns regarding the chimera model of T cell differentiation used in the present study are that (*a*) the donor bone marrow inoculum was contaminated with mature postthymic T cells, and (*b*) the postthymic environment is complex, containing both donor and host elements that could selectively expand donor-restricted T cell subpopulations. While the T cell–depleted donor bone marrow inoculum in the present study may have been contaminated with

a small but undetectable number of residual, mature postthymic T cells, it is difficult to understand how they would be selectively contaminated with class II-restricted L3T4$^+$ CTL and not with class II-restricted L3T4$^+$ Th cells. Similarly, it is difficult to envision postthymic events that would selectively obscure thymic skewing of unprimed class II-restricted L3T4$^+$ CTL without also obscuring skewing of unprimed class II-restricted L3T4$^+$ Th cells. Nevertheless, such concerns cannot be completely excluded and experiments to more directly evaluate them are in progress.

What potential benefit might an animal derive from generating L3T4$^+$ CTL capable of lysing only Ia$^+$ cells? It would be expected that increasing the number and diversity of MHC molecules that CTL use as restricting elements would significantly diminish the likelihood of an animal being a nonresponder to a viral pathogen. It is possible that in the course of a viral infection cells that are ordinarily Ia$^-$ might be induced to become Ia$^+$, since Ia$^-$ cells can be experimentally induced by gamma interferon to express Ia determinants (31). Of interest is the recent finding (32, 33) that most anti-measles virus CTL generated from a patient with multiple sclerosis express the T4$^+$ T8$^-$ Lyt phenotype and are HLA class II restricted. Whether or not class II-restricted L3T4$^+$ CTL play a role in viral diseases, it would seem reasonable that their activation is tightly regulated so as not to interfere with normal immunity by lysing Ia$^+$ antigen-presenting cells and Ia$^+$ B cells, and that such regulation might be defective in certain autoimmune states. The possibility that the activation of class II-restricted pCTL is actively regulated is currently under investigation.

In conclusion, the present study documents that, in the radiation bone marrow chimera model of T cell differentiation, the chimeric thymus does not dramatically skew the self specificity of class II-restricted, L3T4$^+$ anti-TNP pCTL. These results suggest that functionally distinct, but otherwise similar, helper and cytolytic T cell subpopulations are not equally influenced by the MHC haplotype of the thymus.

Summary

The goals of the present study were: (a) to generate antigen-specific L3T4$^+$ cytolytic T lymphocytes (CTL), (b) to determine their major histocompatibility complex (MHC) restriction specificity, and (c) to assess the influence of thymic MHC determinants on their self specificity. We found that L3T4$^+$ CTL specific for either trinitrophenyl (TNP)-modified self determinants or minor histocompatibility antigens could be generated from Lyt-2$^-$ responder T cells provided that the response cultures were supplemented with supernatants rich in helper factors. Such antigen-specific L3T4$^+$ CTL were Ia-restricted by the criteria that they lysed only Ia$^+$ target cells and that their lysis of Ia$^+$ target cells was specifically inhibited by anti-Ia monoclonal antibodies. The relative frequency of L3T4$^+$ pCTL was found to be only 5–10% of the total anti-TNP pCTL present in the spleens of normal mice. Finally, we utilized radiation bone marrow chimeras to assess the influence of the thymic haplotype on the self-Ia specificity of L3T4$^+$ CTL. Both bulk culture and limiting dilution experiments revealed that the self-Ia specificity of L3T4$^+$ anti-TNP CTL from $F_1 \rightarrow$ parent and A $\rightarrow$ B allogeneic chimeras was not markedly skewed toward the haplotype of the chimeric thymus.

These results contrast with those obtained previously for L3T4$^+$ anti-TNP Th cells and demonstrate that in the radiation bone marrow chimera model of T cell differentiation, the self specificity of Th cells but not pCTL is markedly influenced by the haplotype of the chimeric thymus.

We are grateful to Dr. Laurie Glimcher for generously providing the M12.4.1 and M12.C3 cell lines; Ms. Caroline Harrison for excellent technical assistance; and Drs. William Biddison, Richard Hodes, Susan McCarthy, Stephen Shaw, and Gene Shearer for critically reviewing this manuscript.

Received for publication 9 May 1985.

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