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INTRATHYMIC DIFFERENTIATION OF CYTOTOXIC T LYMPHOCYTE (CTL) PRECURSORS

I. The CTL Immunocompetence of Peanut Agglutinin-Positive (Cortical) and Negative (Medullary) Lyt 123 Thymocytes[1]

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To analyze the developmental and functional interrelationship between cortical and medullary thymocytes, the peanut agglutinin- (PNA) binding capacity was used to separate thymocytes into PNA$^+$ (cortical) and PNA$^-$ (medullary) thymocytes. Virtually, all positively selected PNA$^+$ thymocytes (90% of the overall thymocyte population) expressed the Lyt 123 phenotype, whereas 90% of negatively selected PNA$^-$ thymocytes expressed Lyt 1 alloantigens, about 10% being Lyt 123 thymocytes.

Provided, the requirement of Lyt 1 T helper cells was bypassed by Interleukin 2, a nonspecific mediator of T help, PNA$^+$ Lyt 123 thymocytes mounted cytotoxic T cell responses comparable in magnitude to that of peripheral T cells. Their repertoire included antigenic disparities coded for by the complete MHC complex, H-2K I-A, H-2D, mutational events at H-2K, as well as antigenic disparities expressed on TNP conjugated- and Sendai virus-infected syngeneic cells.

PNA$^-$ Lyt 123 thymocytes represent a highly reactive pool of primary cytotoxic T lymphocyte (CTL) precursors for both alloreactive and H-2-restricted CTL responses. Since PNA$^-$ thymocytes include also Lyt 1 T helper cells, PNA$^-$ responder thymocytes are able to mount autonomously CTL responses. Our data are first to provide direct evidence that Lyt 123 cells represent a common source of alloreactive and H-2-restricted CTL precursors in unprimed lymphocyte populations. Moreover, the apparent immunoincompetence of cortical PNA$^+$ thymocytes is now explained by their lack of T helper cells.

Selection, differentiation, and amplification of developing T lymphocytes take place within the thymus (1–3). On the basis of corticosteroid sensitivity, H-2 and Thy 1 membrane contents, 2 major thymocyte subpopulation have been defined: cortical thymocytes and medullary thymocytes, respectively (4, 5). In regard to immune reactivity and antigenic properties, medullary thymocytes are very similar to peripheral T cells, whereas cortical thymocytes are believed to be yet immature cells unable to express immunocompetence (4–8). The ontogenic relationship between both thymocyte subsets is unclear. On the one hand, it has been suggested that cortical thymocytes reflect a sterile differentiation pathway (5), whereas others have proposed that immunocompetent medullary thymocytes develop from cortical precursor cells (7, 8).

To analyze the developmental and functional interrelationship between cortical and medullary thymocytes, the prime task we aimed at was to separate both cell populations and subsequently to determine the immune reactivity of the individual thymocyte subsets. Peanut agglutinin (PNA)[2] binds to 80 to 90% of thymocytes. These PNA$^+$ thymocytes express high Thy 1, low H-2 membrane contents, and the TLa antigen (9–13). Moreover, PNA$^+$ thymocytes are corticosteroid sensitive. In contrast, PNA$^-$ thymocytes compare well with immunocompetent cortisone-resistant thymocytes (9–13). It has therefore been inferred that PNA$^+$ thymocytes represent the immature cortical population of the thymus, whereas the PNA$^-$ cells essentially represent medullary thymocytes. Consequently, we positively selected by cell affinity chromatography PNA$^+$ (cortical) thymocytes, and purified PNA$^-$ (medullary) thymocytes by using negative selection procedures. To bypass the requirement of antigen-specific T helper cells for the induction of alloreactive or H-2-restricted cytotoxic T lymphocytes (CTL), the thymocyte subset to be induced was sensitized in the presence of the previously described (14, 15) nonspecific mediator of T help termed Interleukin 2 (Il-2). Here we report that on the level of CTL precursors Lyt 123$^+$ PNA$^+$ (cortical) thymocytes express immune competence and a repertoire similar to that of peripheral, mature T cells. Although PNA$^+$ (cortical) thymocytes apparently lack T helper cells, PNA$^-$ (medullary) thymocytes are rich in Lyt 1$^+$ T cells and contain PNA$^-$ Lyt 123$^+$ CTL precursors.

MATERIALS AND METHODS

Mice. CBA, BALB/c, C57BL/6 mice were obtained from OLAC, Ltd., Shaw's Farm, Blackthorn, England. The C57BL/6 congenic strain C57BL/6 Lyt^a/Boy, abbreviated B6/Lyt 1.1, was kindly provided by Dr. E. Boyse (Memorial Sloan-Kettering Cancer Institute, New York, NY). The C57BL/6-Hz 1

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[1] This work was supported by the SFB 107 and the Stiftung Volkswagenwerk.

[2] Abbreviations used in this paper: CTL, cytotoxic T lymphocytes; Il-2, Interleukin 2; LAF, lymphocyte-activating factor; MHC, major histocompatibility complex; PNA, peanut-agglutinin; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid.

mutants were obtained from Dr. J. Klein, Tübingen, West Germany.

Antisera. Monoclonal anti-Lyt 1.1 antibodies were kindly provided by Dr. I. F. C. McKenzie (Melbourne, Australia). Monoclonal Lyt 2.2 antibodies were kindly provided by Dr. U. Hämmerling (Sloan-Kettering Institute). Conventional anti-Lyt 1.2 antiserum was kindly provided by the National Institute of Health, Bethesda, MD. Monoclonal anti-Thy 1 antisera were kindly provided by Dr. P. Lake, University College, London. The sera were all absorbed before use with lymphocytes of the appropriate negative congenic strains, and the specified activity of each serum could be absorbed with cells of the appropriate positive congenic strains. Rabbit anti-PNA antibodies were obtained after immunization with PNA purified as described by Irlé (16). Prior use in a complement- (C) dependent cytotoxicity assay, the antiserum was extensively absorbed with thymocytes. Thereafter, its titer was 1:128 toward PNA-binding thymocytes. PNA receptor-positive thymocytes were enumerated by using fluorescein isothiocyanate-labeled PNA from Industrie Biologique Francaise, Clichy, France.

The use of anti-Lyt antisera has been described (14). In short, thymocytes bearing the selectively expressed Lyt cell surface alloantigen were resuspended in the diluted antiserum at a concentration of 10×10^6/ml and incubated at 4°C for 30 min, centrifuged and resuspended in carefully selected nontoxic rabbit C at 1 to 12 dilution, and incubated for 45 min at 37°C. The number of viable cells remaining after treatment was determined by the dye exclusion method.

Positive selection of PNA$^+$ thymocytes. This was performed by cell affinity chromatography according to Irlé *et al.* (13) as described (15). PNA-binding thymocytes were recovered by washing the column with PBS containing 0.15 M galactose. The percent PNA$^+$ cells in the eluate was tested by direct immunofluorescence and proved to be >98%.

Negative selection of PNA$^-$ thymocytes. Thymocytes not adhering to the "PNA column" were still contaminated with about 30 to 50% PNA$^+$ cells as assessed by direct immunofluorescence. After treatment with a rabbit anti-PNA antiserum plus C, the percent of viable PNA-binding thymocytes was reduced to <5%.

Cortisone-resistant thymocytes. Mice were injected i.p. with 2 mg of hydrocortisone acetate 48 hr before sacrifice. Two hours before sacrifice, mice were injected i.v. with Evans blue in order to locate parathymic lymphnodes. In excising the thymuses, care was taken not to co-excise lymph nodes. The yield from the thymuses of treated animals was 5 to 8% of normal controls.

Semipurified Il-2. Il-2 was prepared either from Con A-stimulated lymphocyte culture supernatants (A) or from secondary MLC supernatants (B) as described (14, 15). A): Spleen cells from CBA mice were cultured (7×10^6/ml) in medium without FCS in the presence of 1 μg Con A/ml (Pharmacia, Sweden). After 20 hr, the supernatant was concentrated by pressure filtration (cutoff 10,000 daltons). The concentrate was applied on a previously calibrated Sephadex G-100 column. Effluent fractions were tested for their capacity to sustain growth of CTL *in vitro* (14). Active fractions were pooled, concentrated, and kept frozen at −20°C. B): MLC-primed responder cells (3×10^6) were restimulated with 2×10^6 stimulator cells in medium containing 1% FCS. After 30 hr, the supernatant was harvested and concentrated 10-fold by pressure dialysis. The concentrate was semipurified on Sephadex G-100.

Generation and assay of CTL. Alloreactive CTL: Responder cells (4×10^6) were cultured with stimulator cells (1×10^6) in 2 ml of Dulbecco's modified Eagle's medium (DEM) supplemented with 10 mM HEPES[2] 5×10^{-5} M 2-mercaptoethanol and 5% FCS using multiculture plates (Linbro FB24, Tc, Linbro Chemical Company, New Haven, CT) as described (14). Before culture, stimulator cells were x-irradiated with 2000 rads (Philips machine RT 200, Fa. Müller, Frankfurt, West Germany) at a dose rate of 620 rads/min. When indicated, flat-bottom microcultures (Fa. Greiner, Nürtingen, West Germany) were also used.

Trinitrophenyl- (TNP) specific CTL: The method described by Shearer (17) has been used. Briefly, x-irradiated (2000 rads) splenic lymphocytes (10^7) were incubated for 10 min at 37°C in PBS containing 10 mM trinitrobenzene sulfonic acid (TNBS; Eastman Organic Chemicals Div., Rochester, NY). TNP-conjugated stimulator cells were co-cultured with syngeneic responder cells.

Sendai virus-specific CTL: The method described by Jung *et al.* (18) has been used. Briefly, x-irradiated splenic cells were coated with Sendai virus by incubation of 1×10^7 cells with 200 hemagglutination units of β-propiolactone-inactivated Sendai virus suspensions (Lot. No. 138-1, Connaught Lab., Ltd., Toronto, Canada). Responder cells were cultured with Sendai virus-coated stimulator cells as specified. Cytotoxic activity was assayed on Sendai virus-coated tumor cells syngeneic to the responder cells.

Target cells: P815(H-2^d), EL$_4$(H-2^b), and LS(H-2^k) tumor cells were propagated *in vitro.*

Cytotoxicity assay: Graded numbers of viable cells harvested from MLC were incubated for 4 hr with a constant number (1×10^4) ^{51}Cr-labeled target cells as described (14). Percent specific ^{51}Cr-release was calculated according to the formula:

$$\% \text{ Specific lysis} = \frac{\begin{matrix}{}^{51}\text{Cr-release by immune cells} \\ - \text{ } {}^{51}\text{Cr-release by responder cells cultured without stimulator cells}\end{matrix}}{\begin{matrix}\text{Maximal } {}^{51}\text{Cr-release} \\ - \text{ } {}^{51}\text{Cr-release by responder cells cultured without stimulator cells}\end{matrix}} \times 100$$

For each lymphocyte population tested, a dose-response curve was established. By plotting percent lysis obtained *vs* the log of the ratio of attacker to target cells used, the number of attacker cells could be defined that lysed within 4 hr 50% of 1×10^4 target cells. 1 lytic unit (LU) was arbitrarily defined as the number of lymphoid cells necessary to lyse 50% of the target cells within the assay time.

RESULTS

Thymocytes are apparently poor in helper T cell precursors, as indicated by the observation that their CTL responsiveness can be greatly enhanced by addition of antigen-specific T helper cells (15, 19, 20). The requirement for antigen-specific T helper cells in turn can be substituted by addition of Lyt 1$^+$ helper T cell-derived mediator of T help termed Interleukin 2 (Il-2) (15, 21, 22). The functional activity of Il-2 is neither H-2 restricted nor antigen specific (22, 23). As can be seen in Figure 1, optimal concentrations of semipurified Il-2 allow thymocytes to mount alloreactive as well as H-2-restricted Sendai virus or TNP-specific CTL responses comparable in magnitude to those obtained with splenic responder cells. These results confirmed previous reports indicating that thymocytes are rich in CTL precursors, the antigen-specific activation of which is profoundly dependent on exogeneously added Il-2 (15, 19–22).

Characterization of thymic responder cells. To determine whether the CTL precursors within thymocytes were derived from the medullary (PNA$^-$) or cortical (PNA$^+$) thymocyte

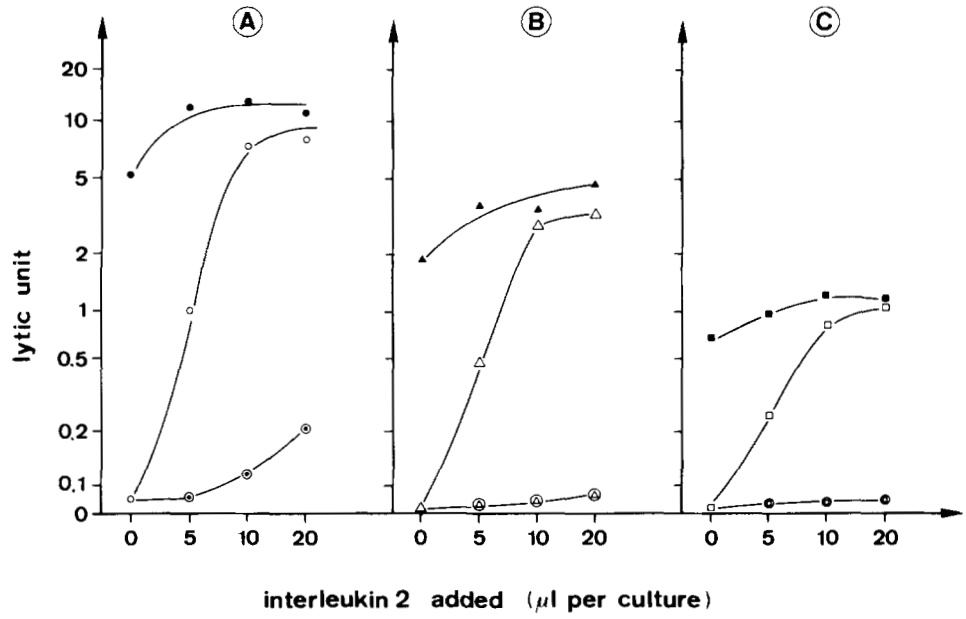

Figure 1. Comparison of the dependency of CTL responsiveness of CBA mouse-derived thymocytes and splenic cells of exogeneously added semipurified Il-2. *A*, CTL responsiveness to H-2^d alloantigen; *B*, CTL responsiveness to TNP-conjugated syngeneic spleen cells; *C*, CTL responsiveness to Sendai virus-conjugated syngeneic spleen cells. Responder cells (3×10^5) plus stimulator cells (5×10^5) were cultured in the presence of a graded amount of semipurified Il-2 in flat bottom microtiter cultures. After 6 days individual cultures were harvested and tested for lytic activity against respective targets in a 4-hr ^{51}Cr-release assay. Background lysis did not exceed 29%. From the individual dose response curves lytic units were calculated. *A*, ●——●, lytic activity of splenic responder cells toward P815 (H-2^d) targets; ○——○, lytic activity of thymocytes toward P815 (H-2^d), and ⊙——⊙, toward EL_4 (H-2^b) targets. *B*, ▲——▲, lytic activity of splenic responder cells toward TNP-conjugated LS (H-2^k) targets; △——△, lytic activity of thymocytes toward TNP-conjugated and ◬——◬, normal LS (H-2^k) targets. *C*, ■——■, lytic activity of splenic responder cells toward Sendai virus-conjugated LS (H-2^k) targets; □——□, lytic activity of thymocytes toward Sendai virus-conjugated and ⧈——⧈ normal LS (H-2^k) targets.

subset, PNA^+ cells within thymocytes were positively selected by cell affinity chromatography (see *Materials and Methods*). Thymocytes adhering to and specifically eluted from the "PNA column" consisted >98% of PNA^+ thymocytes as judged by direct immunfluorescence (Table I). "Nonadhering" thymocytes consisted of about 30 to 50% PNA^+ cells. By treatment with anti-PNA antibodies plus C, a PNA^- thymocyte population was obtained essentially devoid of contaminating PNA^+ cells (Table I).

Next, the Lyt phenotype of unfractionated thymocytes, PNA^+ and PNA^- thymocytes, was investigated and compared with that of cortisone-resistant thymocytes. In agreement with recent data of Mathieson *et al.* (24, 25), we noted that normal thymocytes contained about 10% of cells expressing the Lyt 1 phenotype, whereas 90% of thymocytes were Lyt 123^+ cells. No evidence for the existence of Lyt 23^+ thymocytes was obtained, provided contamination by parathymic lymph node cells was excluded (Table I). The interesting finding, however, was that positively selected PNA^+ thymocytes expressed all the Lyt 123^+ phenotype. This finding allows positive selection of thymic Lyt 123^+ cells by using the PNA marker. Another point of interest was the fact that the majority (about 90%) of PNA^- cells expressed the Lyt 1 phenotype, about 10% being Lyt 123^+ cells (Table II). The existence of PNA^- Lyt 123^+ thymocytes was already indicated by the finding that within cortisone-resistant thymocytes, the percent of Lyt 123^+ cells exceeded that of PNA^+ thymocytes (Table II).

TABLE I

Fractionation of CBA-thymocytes into PNA^+ and PNA^- cells by cell affinity chromatography[a] and treatment with anti-PNA antibodies plus C

Cells	Recovery	Direct Immune Fluorescence	
		PNA^+	PNA^-
	%	%	
Normal thymocytes	100	85	10
Cells specifically eluted[b] from column	60	>99	<1
Cells nonadhering to column	8	25	75
Nonadhering cells after treatment with anti-PNA antibody + C	0.5–2	<3	>97

[a] Representative experiment out of 11 performed.

[b] Thymocytes from 4-week-old mice were fractionated by cell affinity chromatography with the use of PNA as ligand. Specifically adherent cells were eluted in the presence of galactose (15).

Cytotoxic T lymphocyte responsiveness of PNA^+ Lyt 123^+

TABLE II

Lyt phenotype of PNA^+, PNA^-, and cortisone-resistant thymocytes

Cell Population[a] of B6/Ly 1.1 Thymocytes	% PNA^+[b]	Lyt Phenotype (% Cells)[c]		
		Lyt 1^+	Lyt 23^+	Lyt 123^+
Unfractionated	80	<15	<1	>85
PNA^+ cells	>99	<1	<1	>99
PNA^- cells	<3	91	<1	10
Cortisone-resistant cells	12	78	<1	22

[a] Thymocytes from 4-week-old mice were fractionated by cell affinity chromatography by using PNA as ligand. Specifically adherent cells were eluted in the presence of galactose. Cortisone-resistant thymocytes were obtained from mice injected 2 days previously with 2.5 mg hydrocortisone.

[b] Estimated by using FITC-conjugated PNA.

[c] Quantitated by using C-dependent cytotoxicity assay as described in *Materials and Methods*.

thymocytes. Positively selected PNA$^+$ Lyt 123 thymocytes on its own were unable to mount alloreactive CTL. However, in the presence of Il-2, highly reactive CTL could be induced (Fig. 2). A characteristic feature of CTL induction from PNA$^+$ Lyt 123$^+$ thymic responder cells was that peak response occurred as late as day 5 to 7. The data given in Table 3 provide evidence that PNA$^+$ thymic responder cells contained CTL precursors able to mount both alloreactive and H-2-restricted TNP-specific CTL responses. Although there existed an absolute Il-2 requirement for the phenotype expression of CTL-immunocompetence, in the presence of Il-2, efficient CTL responses could be elicited even towards stimulator cells differing only at D, K, I-A, or by mutational events at H-2K (Table III). The latter finding is interesting in view of previously reported data on the specific role of H-2I region determinants in generation of cytotoxic responses from thymic responder cells (26). In summary, the results presented so far contrast with the view that PNA$^+$ thymocytes are immunoincompetent. The phenotypic lack of immunocompetence of PNA$^+$ thymocytes appeared rather to be due to the absence of mature Lyt 1$^+$ T cells required for the production of Il-2, which in turn acts as a second signal to initiate the triggering events of clonally distributed PNA$^+$ Lyt 123$^+$ CTL precursors (*vide infra*).

CTL responsiveness of PNA$^-$ thymocytes. Within PNA$^-$ thymocytes, it is the PNA$^-$ Lyt 123$^+$ subset that provides the pool of CTL precursors (Table IV). In comparing the CTL responsiveness between PNA$^-$ and PNA$^+$ thymocyte subsets, 2 significant differences become apparent. First, PNA$^-$ cells are, like cortisone-resistant cells, able to mount autonomously both alloreactive and H-2-restricted TNP-specific CTL; that is, their CTL responsiveness is not absolutely dependent on exogeneously added Il-2. Il-2 only enhances the magnitude of the CTL response generated (Table V). Second, unlike PNA$^+$ Lyt 123$^+$ thymocytes, PNA$^-$ Lyt 123$^+$ thymocytes mount peak CTL responses earlier in time (Fig. 2). In comparing the results given in Tables II and III, which indicate that only a minority of about 10% of the input PNA$^-$ cells provided the pool of cells out of which CTL precursors are activated, the magnitude and early response depicted in Figure 2 is surprising. Our conclusion at present is that PNA$^-$ Lyt 123$^+$ thymocytes represent a pool of mature, highly reactive Lyt 123$^+$ CTL precursors.

Helper cell activity of PNA$^+$ and PNA$^-$ thymocytes. Two further lines of experimental evidence support our conclusion that PNA$^+$ thymocytes contain CTL precursors yet lack functional active T helper precursor cells, whereas PNA$^-$ cells and cortisone-resistant cells are rich in functional active T helper cells. First, polyclonal activation of PNA$^-$ and cortisone-resistant thymocytes yields in culture supernatant high Il-2 activity.

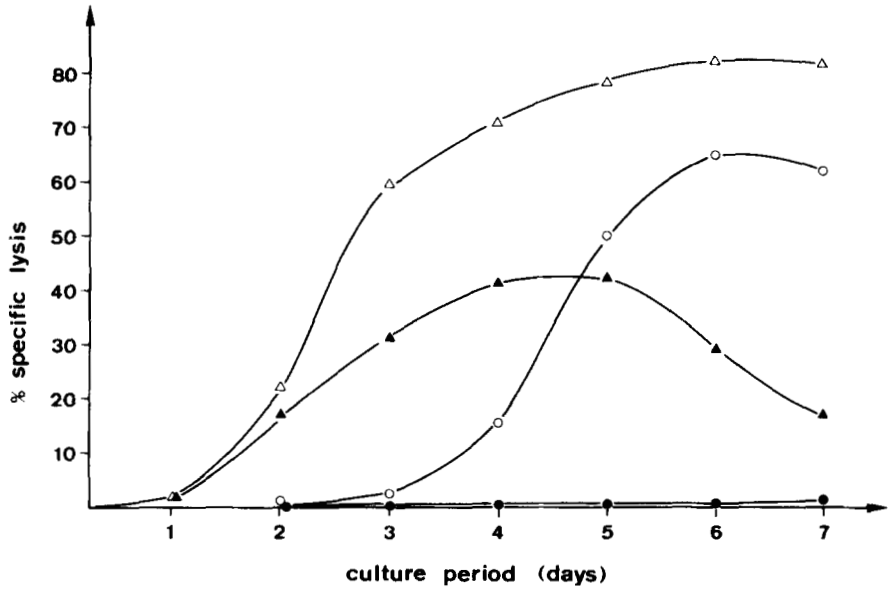

Figure 2. CTL responsiveness of PNA$^+$ and PNA$^-$ thymocytes in the presence or absence of Il-2. PNA$^+$ responder thymocytes (>99% PNA$^+$, >99% Lyt 133$^+$ cells) were prepared from B6/Ly 1.1 mice. Responder cells (0.5×10^6) were co-cultivated with irradiated H-2^d stimulator cells (1×10^6) in replicate microwell cultures in the absence (●——●) or presence (○——○) of 10 μl semipurified Il-2. At the time points given, cells from 2 replicate cultures were pooled and the cytolytic activity generated was tested against ^{51}Cr-labeled P815 (H-2^d) targets. PNA$^-$ responder thymocytes (<3% PNA$^+$; 85% Lyt 1$^+$; 12% Lyt 123$^+$) were prepared from the same B6/Ly 1.1 mice. Responder cells (0.5×10^6) were co-cultivated with H-2^d stimulator cells as described above in the presence (△——△) or absence (▲——▲) of semipurified Il-2. Background lysis of P815 (H-2^d) target cells did not exceed 26% during the 4-hr cytotoxicity test.

TABLE III

PNA$^+$ (cortical) thymocytes exhibit immunocompetence on the level of CTL precursors

Responder Thymocytes[a] (>99% PNA$^+$)	Stimulator Cells	Antigenic Disparities	Il-2 (μl/Culture)	Target Cells	% Specific Lysis[b] 20:1	5:1	1:1
CBA	BALB/c	All of H-2 plus minor H	—	P815	2	0	1
			75		72	36	22
C57BL/6	B10.A (4R)	H-2K, I-A	—	LS	1	1	0
			75		27	10	4
Hz 1	C57BL/6	H-2K^{ha} → H-2K^b	—	EL$_4$	3	1	0
			75		55	26	14
BALB/c	B10.HTG	H-2D	—	EL$_4$	0	0	0
			75		69	49	20
CBA	CBA-TNP	TNP	—	LS-TNP	0	0	0
			75		36	18	1
BALB/C	BALB/c-Sendai virus	Sendai virus	—	P815	0	0	0
			75	Sendai	24	7	3

[a] PNA$^+$ thymocytes (4×10^6) were cultured in Linbro well plates in the presence or absence of Il-2 plus 2×10^6 stimulator cells for 5 to 6 days.
[b] Ratio of CTL to target cells during the 4-hr cytotoxicity test. Background lysis of target cell did not exceed 27%.

TABLE IV

Lyt phenotype of CTL precursors within PNA⁻ C57BL/6 thymocytes

Responder Cells[a]	Treatment	Stimulator Cells	Il-2 (10 μl)	% Specific Lysis[b]			
				25:1	5:1	1:1	0.2:1
Unfractionated		H-2^d	+	73	54	18	
PNA$^+$ (>99%)		H-2^d	+	53	27	12	
PNA$^-$ (<3% PNA$^+$)	NMS plus C	H-2^d	+	73	68	33	6
PNA$^-$	Anti-Thy plus C	H-2^d	+	0	0	0	
PNA$^-$	Anti-Lyt 1.2 plus C	H-2^d	+	4	1	0	
PNA$^-$	Anti-Lyt 2.2 plus C	H-2^d	+	2	1	0	

[a] Thymocytes of mice were positively selected for PNA$^+$ cells and negatively selected for PNA$^-$ cells. Aliquots of PNA$^-$ cells were treated either with NMS plus C, with anti-Lyt 1.2 serum plus C, or with anti-Lyt 2.2 serum plus C. Viable cells (0.3×10^6) were co-cultivated in microtiter wells with 0.5×10^6 stimulator cells plus Il-2. After 5 days cytotoxicity generated was assayed toward P815(H-2^d) targets in a 4-hr ^{51}Cr-release test. Background lysis was 11%.

[b] Ratio of CTL to target cells.

TABLE V

Effect of Il-2 on CTL responsiveness of PNA$^+$, PNA$^-$, and cortisone-resistant thymocytes

Responder Cells[a] (CBA Thymocytes)	Stimulator Cells	Il-2 (10 μl)	% Specific Lysis							
			P 815		EL$_4$		TNP-LS		LS	
			10:1	2:1	10:1	2:1	10:1	2:1	10:1	2:1[b]
PNA$^+$ (>99%)	BALB/c	−	0	0	0	0	N.D.		N.D.	
		+	54	32	13	4				
	TNP-CBA	−	N.D.		0	0	1	0	0	0
		+			6	4	59	31	8	4
PNA$^-$ (<5% PNA$^+$)	BALB/c	−	38	21	8	2	N.D.		N.D.	
		+	62	37	24	6				
	TNP-CBA	−	N.D.		N.D.		44	13	9	2
		+					59	28	12	5
Cortisone-resistant thymocytes (18% PNA$^+$)	BALB/c	−	42	29	N.D.		N.D.		N.D.	
		+	71	38						
	TNP-CBA	−	N.D.		N.D.		54	33	12	4
		+					62	29	17	8

[a] PNA$^+$ thymocytes were prepared by positive selection, PNA$^-$ thymocytes by negative selection and cortisone-resistant thymocytes by *in vivo* application of hydrocortisone. The number of PNA$^+$ cells in the individual cell populations was assessed by direct immunofluorescence. Responder and stimulator cells were co-cultivated in the presence or absence of Il-2 in flat bottom microtiter plates. Cytotoxic activity was assayed after 5 days toward the target cells listed. Background lysis did not exceed 25%.

[b] Ratio of CTL to target cells.

[c] N.D., not done.

TABLE VI

Production of Il-2 by subpopulations of thymocytes

Il-2 Prepared from[a]	Day 3 Response of[b] H-2^k Anti H-2^d-Primed Cells
	LU/culture
No cells (medium plus 1 μg Con A/ml)	<0.5
CBA-spleen cells	31
CBA-PNA$^+$ thymocytes	<0.5
CBA-PNA$^-$ thymocytes	24
CBA-cortisone-resistant thymocytes	31

[a] Il-2 was prepared by stimulating 5×10^6 cells/ml with 1 μg Con A for 24 hr as described (14).

[b] To test for Il-2 production MLC primed H-2^k anti H-2^d cells (day 7) were distributed into Linbro wells (1×10^5 viable cells) and cultured in the presence of 20% (v/v) of culture supernatant for 3 days as described (14). Thereafter cytolytic activity was determined toward ^{51}Cr-labeled P815 cells.

On the other hand, PNA$^+$ thymocytes fail to produce Il-2 (Table VI). Second, when graded numbers of mitomycin C-treated cortisone-resistant thymocytes were added to PNA$^+$ thymic responder cells and the cell mixture was cultured for 6 days in the presence of stimulator cells, alloreactive CTL were generated (Table VII). Since neither of the responder cells alone mounted a significant CTL response, this type of experiment suggested that mitomycin C-treated cortisone-resistant cells are rich in T helper cell activity, thereby allowing PNA$^+$ CTL precursors to be induced.

DISCUSSION

The experimental results described here relate to the immunocompetence of thymocyte subsets as defined by their PNA-binding capacity and their Lyt phenotype. PNA$^+$ thymocytes comprising about 80 to 90% of the overall cell population are found to express the Lyt 123$^+$ phenotype. This finding allows positive selection of virtually pure Lyt 123$^+$ thymocytes. In contrast, the great majority (about 90%) of thymic PNA$^-$ cells are Lyt 1$^+$ thymocytes, yet PNA$^-$ cells also include a minority (about 10%) of Lyt 123$^+$ thymocytes. Positively selected PNA$^+$ Lyt 123 thymocytes, so far equated with cortical thymocytes (9–13), were found to express complete CTL precursor immunocompetence, the phenotypical manifestation of which was strictly dependent on the presence of T helper cells. The requirement for T helper cells in turn could be substituted by Il-2, a Lyt 1$^+$ T helper cell-derived nonspecific mediator of T help (15, 21).

Unlike PNA$^+$ Lyt 123$^+$ thymocytes, PNA$^-$ thymocytes, so far

TABLE VII

Effect of adding mitomycin C-treated "cortisone-resistant" thymocytes on CTL responsiveness of PNA+ thymocytes

Responder Cells[a]		F_1 (CBA × BALB/c) Stimulator Cells (8×10^5)	% Specific Lysis of P815 Targets[b]		
PNA+ CBA-thymocytes (98% PNA+)	Cortisone-resistant CBA-thymocytes (62% PNA-)		20:1	5:1	1:1
	4×10^5	+	54	32	8
	$[4 \times 10^5]_{Mito}$	+	1	0	0
4×10^5		+	2	1	0
4×10^5	4×10^5	+	32	18	4
4×10^5	2×10^5	+	47	23	14
4×10^5	0.5×10^5	+	19	8	3
4×10^5	0.1×10^5	+	7	1	0

[a] PNA+ thymocytes were obtained by positive selection. Cortisone-resistant thymocytes were obtained by *in vivo* application of hydrocortisone. The number of PNA+ and PNA- cells was assessed by direct immunofluorescence. Responder and stimulator cells were cultured together in flat bottom microtiter plates for 6 days and thereafter assayed for cytolytic activity.

[b] Ratio of CTL to target cells.

equated with medullary thymocytes (9–13), expressed immunocompetence on its own. PNA- cells were found to be rich in T helper cells. Moreover, upon polyclonal stimulation, PNA- thymocytes were able to produce Il-2.

To date, T cell-specific anti-Lyt alloantisera have allowed negative selection of T cell subpopulations. Using this approach, it has been demonstrated that Lyt 1+ cells are able to help humoral or cellular immune responses (27–29), whereas T cells expressing the Lyt 23+ phenotype elicit suppressor activity (30) and comprise alloreactive and H-2-restricted killer cell precursors (27, 31). The functional activity of Lyt 123+ T cells has been less well characterized because this T cell subset cannot be isolated by negative selection using anti-Lyt antisera and C. Nevertheless, it has been inferred from indirect evidence, that Lyt 123+ T cells contain all primary precursors for Lyt 1+ T helper cells and Lyt 1+ suppressor inducer (32, 33), and the primary precursors for alloreactive and H-2-restricted CTL (32, 34). Using positively selected Lyt 123+ thymocytes, we now provide direct evidence that Lyt 123+ cells are the common source of alloreactive and H-2-restricted CTL precursors in unprimed and unselected lymphocyte populations (Tables I–III; Fig. 2). In a forthcoming report (H. Wagner *et al.*, manuscript in preparation), we will describe that by differentiation from antecedent Lyt 123+ PNA+ thymocytes, both alloreactive and H-2-restricted PNA- Lyt 123+ CTL can be induced *in vitro*.

Positively selected Lyt 123+ thymocytes have proven to be useful for analyzing the essential role of Lyt 1+ T helper cells during the antigen-specific activation of Lyt 123+ CTL precursors. As described elsewhere (32), in the presence of a product from I-A-positive macrophages, termed lymphocyte-activating factor (LAF; synonym Interleukin 1), antigen-specific activation of splenic Lyt 1+ T cells results in the release of the mediator of T help, i.e., Il-2, in the presence of which Lyt 123+ CTL precursors are activated by antigen and differentiate into alloreactive or H-2-restricted cytotoxic effector cells (Fig. 2, Table III).

The necessity of T–T cell interactions during the induction of CTL from Lyt 123+ cells explains also the apparent immunocompetence of PNA- or of cortisone-resistant thymocytes. PNA- cells contain high numbers of Lyt 1+ thymocytes (Table II). PNA-, Lyt 1+ cells can confer helper activity (Table VII). Furthermore, PNA- thymocytes produce Il-2 (Table VI). Since there is no conversion of Lyt 1+ to Lyt 123+ cells (thymectomized B mice given Lyt 1+ cells do not generate Lyt 123+ cells (35)), we assume that the CTL precursor pool within PNA- thymocytes resides in the PNA- Lyt 123+ thymocyte subset. Since, on the other hand, the concomitant PNA- Lyt 1+ may either act by inducing Lyt 1+ T helper cells from the PNA- Lyt 123+ thymocyte subset (33) or act *per se* as helper cells, sufficient T help is available from within to allow triggering of PNA- Lyt 123+ cells programmed to develop into CTL.

The necessity of T–T cell interactions for the induction of CTL from Lyt 123+ responder cells also explains the apparent lack of immunocompetence of PNA+ thymocytes. Positively selected PNA+ thymocytes obviously are devoid of Lyt 1+ T helper cells or Lyt 1+ T cells inducing T helper cells from Lyt 123+ T helper precursors. The results described here do not correspond with the view that PNA+ (cortical) thymocytes reflect a sterile T cell differentiation pathway (5). In regard to this latter point, it is known that thymocytes turn over every 5 to 7 days (36, 37). There is also good evidence that per day only 2×10^6 thymocytes (38), composed of approximately 70% Lyt 1+ and 30% Lyt 123+ cells (39), migrate out of the thymus. Therefore, it follows that most PNA+ thymocytes must die, presumably *in situ*. Since on the cellular level 1 out of 10^4 PNA+ Lyt 123+ thymocytes represents an alloreactive or TNP-specific CTL precursor cell as determined by "limiting dilution" frequency analysis (R. Bartlett *et al.*, unpublished results), it follows either that potentially immunocompetent PNA+ cells are included in the PNA+ cells being turned over, or that the minority of potentially immunocompetent PNA+ cells is selected by yet unknown mechanisms. As a consequence, such cells could be emigrating directly to the periphery or to the thymic medulla. The latter notion is supported by the finding that the calculated CTL precursor frequency in PNA- Lyt 123+ thymocytes exceeds by 10- to 20-fold that within PNA+ Lyt 123+ thymocytes (unpublished data). That immunocompetent PNA+ Lyt 123+ thymocytes are yet immature is also indicated by the observation that the time period required to differentiate into CTL appears to be longer compared with that of PNA- Lyt 123+ cells (Fig. 2). Another interesting aspect relates to the presently held view that the thymus is instrumental in generating T cell immunocompetence (1–3) and in selecting self-restricted CTL precursors (3). Surprisingly, in the presence of Il-2 plus alloantigen, lymphocytes from thymus-deficient (nu/nu) mice differentiate into alloreactive CTL both *in vitro* (40) and *in vivo* (41). This finding raises the possibility that the thymus-dependent, rate-limiting step for the generation of CTL immunocompetence is operating on the level of Lyt 1+ T helper (inducer) cells, whereas CTL precursors already exist on a prethymic level. This reasoning would explain the lack of T helper cells within PNA+ thymocytes. If, on the other hand, the thymic major histocompatibility complex (MHC) is selecting self-restricted CTL precursors (4), the PNA+ Lyt 123+ thymocytes shown here to be immunocompetent and endowed with a CTL precursor repertoire similar to that of peripheral T cells must have already passed the postulated intrathymic selection process.

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