

ALLOREACTIVE AND H-2-RESTRICTED Lyt 23
CYTOTOXIC T LYMPHOCYTES DERIVE FROM A
COMMON POOL OF ANTECEDENT Lyt 123
PRECURSORS*

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The use of T cell-specific anti-Lyt alloantisera has brought about recognition of phenotypically distinct T cell subsets involved in positive and negative regulation of the immune system (1-8). Based on negative selection of Lyt 1 and Lyt 23 T cell subpopulations, there is compelling evidence that the former act as helper-effector T cells (1-4) or as suppression inducers (3-5), whereas the latter comprise alloreactive and H-2-restricted cytotoxic T lymphocyte (CTL) precursors (1, 6-9) as well as suppressor T cells (1, 4, 5). Because conversion of Lyt 23 cells to Lyt 1 cells (and vice versa) does not occur (10), their relation to one another appears to be entirely communicative.

The majority of peripheral T cells express, however, the Lyt 123 phenotype (1). Because it is difficult to positively select Lyt 123 cells, the present information on their functional potential is less clear (1, 6, 11, 12).

With peanut agglutinin-receptor-positive (PNA⁺) thymocytes as responder cells, the data presented here provide direct evidence that both alloreactive and H-2-restricted Lyt 23 cytotoxic T-effector cells differentiate from antecedent Lyt 123 T cells.

Materials and Methods

Mice. CBA, BALB/c, and C57BL/6 mice were obtained from OLAC Ltd., Shaw's Farm, Blackthorn, England. The breeding stock of the C57BL/6 congenic strain C57BL/6 Lyt^a/Boy (B6/Lyt 1.1) was kindly provided by Dr. E. Boyse, Sloan-Kettering Institute for Cancer Research, New York. The C57BL/6-Hz 1 mutants were bred at the Max-Planck-Institut, Tübingen, Federal Republic of Germany.

Antisera. Monoclonal anti-Lyt 1.1 antibodies were kindly provided by Dr. I. F. C. McKenzie (Austin Hospital, Melbourne, Australia), and monoclonal Lyt 2.2 antibodies by Dr. U. Hämmerling (Sloan-Kettering Institute for Cancer Research). Conventional anti-Lyt 1.2 antiserum was kindly provided by the National Institutes of Health, Bethesda, Md. The use of the anti-Lyt antisera has been described (9). Rabbit anti-peanut agglutinin (PNA) antibodies were obtained after immunization with purified PNA as described (9). Before use in a complement-

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dependent cytotoxicity assay, the antiserum was extensively absorbed with thymocytes. PNA⁺ thymocytes were enumerated by using fluorescein isothiocyanate (FITC)-labeled PNA from Industrie Biologique Française, Clichy, France.

Positive Selection of PNA⁺ Thymocytes. The positive selection of PNA⁺ thymocytes was performed by cell affinity chromatography as described elsewhere (9).

Negative Selection of PNA-Receptor-Negative (PNA⁻) Thymocytes. Thymocytes that did not adhere to the PNA-column were still contaminated with ~30–50% PNA⁺ cells as assessed by direct immunofluorescence (9). After treatment with a rabbit anti-PNA antiserum plus complement (C), the percentage of viable PNA-binding thymocytes was reduced to <5%.

Preparation of Thymocytes. 2 h before killing, mice were injected intravenously with Evans blue to locate parathymic lymph nodes while excising the thymus.

Semipurified Interleukin-2 (Il-2). Il-2 was prepared from concanavalin A-stimulated lymphocyte culture supernates and partially purified as described (13).

In Vitro Generation and Assay of CTL

ALLOREACTIVE CTL. Responder cells (4×10^6), were cultured with x-irradiated (2,000 rad) stimulator cells (1×10^6) in Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum (Seromed, Munich, Federal Republic of Germany) in 2-ml cultures in multiculture plates (Linbro FB24; Tc; Linbro Chemical Co., Hamden, Conn.) as described elsewhere (13).

TRINITROPHENYL (TNP)-SPECIFIC CTL. The method described by Shearer (14) was used for TNP-specific CTL.

SENDAI VIRUS-SPECIFIC CTL. The method described by Jung et al. (15) has been used for Sendai virus-specific CTL.

CYTOTOXICITY ASSAY. Graded numbers of viable cells harvested from mixed lymphocyte cultures were incubated for 4 h with a constant number (1×10^4) ⁵¹Cr-labeled target cells as described elsewhere (9).

Results and Discussion

As reported elsewhere (16) and depicted in Table I, virtually all PNA⁺ thymocytes express the Lyt 123 phenotype, whereas the majority of PNA⁻ thymocytes are Lyt 1 cells, with a minority of ~10% PNA⁻ Lyt 123 cells. The experiments described in Table II establish that positively selected PNA⁺ thymocytes, all of which express the Lyt 123 phenotype, can be induced to differentiate in vitro into CTL, provided the Lyt 1 T cell-derived (13) mediator of T help, Il-2, is added to the culture. This refers to both CTL responses toward antigenic disparities expressed by completely allogeneic stimulator cells, stimulator cells that differ by mutational events of the H-2K molecule, and syngeneic cells either infected with Sendai virus or conjugated with TNP. The data given in Table II also indicate that PNA⁺ Lyt 123 thymocytes, which have been

TABLE I
Lyt Phenotype of PNA⁺ and PNA⁻ Thymocytes

Cell populations* of B6/Lyt 1.1 thymocytes	PNA‡	Lyt phenotype§		
		Lyt 1 ⁺	Lyt 23 ⁺	Lyt 123 ⁺
	%		%	
Unfractionated	80	<15	<1	>85
PNA ⁺ cells	>99	<1	<1	>99
PNA ⁻ cells	<3	91	<1	10

* Thymocytes from 4-wk-old mice were fractionated by cell affinity chromatography with PNA as ligand. Specifically adherent cells (PNA⁺ cells) were eluted in the presence of galactose. PNA⁻ cells were obtained after treatment of nonadherent cells incubated with PNA with rabbit anti-PNA antibodies plus C.

‡ Estimated with FITC-conjugated PNA.

§ Quantitated by a complement-dependent cytotoxicity assay as described in Materials and Methods.

TABLE II
PNA⁺ Lyt 123 Thymocytes Exhibit Immunocompetence on the Level of CTL Precursors

Responder thymocytes* (>99% PNA ⁺) (>99% Lyt 123)‡	Stimulator cells	Antigenic disparities	Il-2 (μl per culture)	Target cells	Percent specific lysis§		
					20:1	5:1	1:1
C57BL/6	BALB/c	All of H-2 plus minor histocompatibility antigens	— 75	P815	2 72	0 36	1 22
C57BL/6	B10.A(4R)	H-2K, I-A	— 75	LS	1 47	1 20	0 4
H21	C57BL/6	H-2K ^{ba} → H-2K ^b	— 75	EL ₄	3 53	1 26	0 14
BALB/c	B10.HTG	H-2D	— 75	EL ₄	0 69	0 49	0 20
CBA	CBA-TNP	TNP	— 75	LS-TNP	0 36	0 18	0 1
BALB/c	BALB/c-Sendai virus	Sendai virus	— 75	P815-Sendai virus	0 24	0 7	0 3

* 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 d.

‡ Quantitated in a complement-dependent cytotoxicity assay.

§ Ratio of CTL:target cells during the 4-h cytotoxicity test. Background lysis of target cell did not exceed 27%.

TABLE III
Change of Lyt Phenotype of CTL

CTL precursors* (>99% PNA ⁺) (>99% Lyt 123)	Stimulator cells	Antigenic disparities	Lytic activity at day 5 after treatment with						Lytic activity at day 9 after treatment with					
			NMS‡ plus C		Anti-Lyt 1.1 plus C		Anti-Lyt 2.2 plus C		NMS plus C		Anti-Lyt 1.1 plus C		Anti-Lyt 2.2 plus C	
			20:1§	5:1	20:1	5:1	20:1	5:1	20:1	5:1	20:1	5:1	20:1	5:1
			%						%					
Lyt 1.1-congenic														
C57BL/6	BALB/c	All of H-2	78	34	10	2	3	0	51	24	49	18	1	1
C57BL/6	B10.A(4R)	K, I-A	56	20	23	11	4	0	43	25	40	21	0	0
C57BL/6	H21	K ^{ba}	60	29	18	9	13	6	63	34	60	27	3	0
C57BL/6	C57BL/6-TNP	TNP	52	35	34	18	0	0	58	27	60	19	0	0
C57BL/6	C57BL/6-Sendai virus	Sendai virus	27	13	31	14	0	0	21	9	20	7	0	0

* PNA⁺ Lyt 123 thymocytes were sensitized in the presence of Il-2 toward the stimulator cells listed. A part of the cells was harvested at day 5, subjected to treatment with anti-Lyt antisera plus C, and the surviving cells were tested for lytic activity. At day 9, the same protocol was repeated. If Il-2 was removed by washing the cells at day 5, no change of the Lyt phenotype occurred, and the lytic activity at day 9 was almost zero (data not shown).

‡ NMS, normal mouse serum.

§ Ratio of CTL:target cells during the 4-h ⁵¹Cr-release assay. The ratio refers to viable lymphocyte numbers before treatment with antisera plus C.

equated with cortical cells (13, 17), express an antigen repertoire and competence similar to that of peripheral T cells (16). A critical information is given in Table III. Here the Lyt phenotype of PNA⁺ Lyt 123 responder cells sensitized in the presence of Il-2 toward alloantigens or toward foreign antigens presented in the context of self major histocompatibility complex (MHC) structures, is analyzed over time. Two points emerge: First, after a sensitization period of 9 d, all CTL generated express the Lyt 23 phenotype regardless of whether they are reactive toward alloantigens, mutant antigens, or foreign antigens presented in the context of self MHC structures. This

TABLE IV
Change of the PNA-binding Activity during the Conversion of Lyt 123 Cells into Lyt 23 CTL

CTL precursors* (>99% PNA ⁺) (>99% Lyt 123)	Stimulator cells	Lytic activity at day 5 after treatment with				Lytic activity at day 9 after treatment with			
		NRS‡ plus C		Anti-PNA plus C		NRS plus C		Anti-PNA plus C	
		20:1§	5:1	20:1	5:1	20:1	5:1	20:1	5:1
		%				%			
Lyt 1.1-congenic C57BL/6	BALB/c	76	31	15	7	49	21	50	28
Lyt 1.1-congenic C57BL/6	C57BL/6-TNP	48	27	12	4	52	29	48	26

* Responder cells were prepared and sensitized as described in the legend to Table III. The cells typed in Table III for their Lyt phenotype were also subjected to treatment with anti-PNA antiserum plus C.

‡ NRS, normal rabbit serum.

§ Ratio of CTL:target cells (see legend to Table III).

loss of Lyt 1 cell surface alloantigens also parallels a loss of the PNA-binding capacity (Table IV). Second, during an intermediate phase of sensitizing culture period, the cytotoxic effector activity is carried out by CTL that still express the Lyt 123 phenotype. This refers to all sensitization protocols tested (Table III).

Taken together, these data provide direct evidence that, in the presence of the Lyt 1 T-helper cell-derived mediator Il-2 (9, 13), Lyt 23 CTL differentiate from antecedent Lyt 123 CTL precursors. A corollary has been recently described for Lyt 1 T-helper-effector and Lyt T-suppressor-inducer cells that have been shown to differentiate also from antecedent Lyt 123 T cells (18). The data therefore establish that it is the Lyt 123 T cell subset from which distinct T cell subsets develop with differential immunological functions. It is yet unclear whether Lyt 123 T cells contain particular sets of precursor cells predetermined to elicit given immunological functions, or whether, under the influence of antigen, the inductive signal provided by Lyt 1 T-helper cells influences the differentiation pathway of yet uncommitted, but clonally restricted, Lyt 123 cells. What can be concluded at present is that the Lyt phenotype of precursor cells does not necessarily correlate with the Lyt phenotype of T effector cells.

In regard to the collaborative relation between Lyt 1 T cells and Lyt 123 or Lyt 23 T cells, two conclusions become apparent: First, in addition to antigen, the collaborative signal provided by Il-2 is essential for the activation of Lyt 123 CTL precursors into Lyt 123 CTL (Table II). Second, Il-2 is essential for the differentiation of Lyt 123 CTL into mature Lyt 23 CTL (Table III). A similar conclusion has been reached by Nagy et al.¹ in analyzing T cell responses to mutant class I antigens. Our data therefore indicate that cytotoxic Lyt 123 T lymphocytes do not represent members of an alternative pathway of T cell activation (19) but rather a transient stage during the differentiation of Lyt 123 CTL precursors into Lyt 23 CTL. We feel this conclusion explains the conflicting data that indicate that CTL responses toward tumor antigens (8) or mutant class I antigens (11) are sometimes carried out by Lyt 123 CTL, whereas others (20) report that the effector cells express the Lyt 23 phenotype.

¹ Nagy, Z., P. Kusnierczyk, and J. Klein. Terminal differentiation of T cells specific for mutant H-2K antigens. Conversion of Lyt T cells into Lyt 2, but not Lyt 1 T cells. Manuscript submitted for publication.

The data presented here establish that the Lyt 123 T cell subset represents a common precursor cell pool for both alloreactive and H-2-restricted CTL. Because Thy-1-negative lymphocytes from athymic (nu/nu) mice already contain CTL precursors (21, 22), we now aim at an analysis on which level of T cell differentiation in the developmental history of the T cell lineage the diversity of separately programmed T subsets is generated.

Summary

If the collaborative requirement of Lyt 1 T helper cells is bypassed by the Lyt 1 T cell-derived mediator of T help, termed Il-2, upon antigenic stimulation, PNA⁺ Lyt 123 thymocytes differentiate into either alloreactive or H-2-restricted PNA⁻ Lyt 23 cytotoxic effector cells. Along the differentiation pathway from Lyt 123 → 23 effector cells, cytolytic activity is carried out by T cells that still express the Lyt 123 phenotype. The data establish that Lyt 23 CTL are produced by differentiation from antecedent Lyt 123 cells.

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References

1. Cantor, H., and E. A. Boyse. 1976. Regulation of cellular and humoral responses by T cell subclasses. *Cold Spring Harbor Symp. Quant. Biol.* **41**:23.
2. Eardley, D. D., J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor. 1978. Immune regulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition. *J. Exp. Med.* **147**:1106.
3. Feldmann, M., P. C. L. Beverly, J. Woody, and I. F. C. McKenzie. 1977. T-T interactions in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursor and amplifier cells. *J. Exp. Med.* **145**:793.
4. Jandiski, J., H. Cantor, T. Tadakuma, D. L. Peavy, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor and helper activities are inherent properties of distinct T-cell subclasses. *J. Exp. Med.* **143**:1382.
5. Herzenberg, L. A., K. Okumura, H. Cantor, V. L. Sato, F. W. Shen, E. A. Boyse, and L. A. Herzenberg. 1976. T cell regulation of antibody responses: demonstration of allotype-specific helper T cells and their specific removal by suppressor T cells. *J. Exp. Med.* **144**:330.
6. Simon, M. M., and B. Abenhardt. 1980. Generation of effector cells from T cell subsets. II. Lyt 123 T cells contain the precursors for all primary cytotoxic effector cells and for cells involved in the regulation of cytotoxic responses. *Eur. J. Immunol.* **10**:334.
7. Burakoff, S. J., R. Finberg, L. Glimcher, F. Lemonnier, B. Benacerraf, and H. Cantor. 1978. The biologic significance of alloreactivity. The ontogeny of T-cell sets specific for alloantigens or modified self antigens. *J. Exp. Med.* **148**:1414.
8. Shiku, H., T. Takahashi, M. A. Bean, L. J. Old, and H. F. Oettgen. 1976. Ly phenotype of cytotoxic T cells for syngeneic tumor. *J. Exp. Med.* **144**:1116.
9. Wagner, H., M. Röllinghoff, K. Pfizenmaier, C. Hardt, and G. Johnschner. 1980. T-T cell interactions during in vitro cytotoxic T lymphocyte (CTL) responses: helper factor from activated Ly 1 T cells recruits allo- or H-2 restricted CTL precursors from the Ly 123 T subset. *J. Immunol.* **124**:1058.
10. Huber, B., H. Cantor, F. W. Shen, and E. A. Boyse. 1976. Independent differentiative pathway of Ly1 and Ly23 subclasses of T cells. Experimental production of mice deprived of selected T-cell subclasses. *J. Exp. Med.* **144**:1128.

11. Wettstein, P. J., B. W. Bailey, L. E. Mobraaten, J. Klein, and J. A. Frelinger. 1979. T-lymphocyte response to H-2 mutants: cytotoxic effectors are Ly-1⁺2⁺. *Proc. Natl. Acad. Sci. U. S. A.* **76**:3455.
12. Kaufmann, H. E., M. Simon, and H. Hahn. 1979. Specific Lyt 123 T cells are involved in protection against *Listeria monocytogenes* and in delayed-type hypersensitivity to listerial antigens. *J. Exp. Med.* **150**:1033.
13. Wagner, H., and M. Rölinghoff. 1978. T-T-cell interactions during in vitro cytotoxic allograft responses. I. Soluble products from activated Ly1⁺ T cells trigger autonomously antigen-primed Ly23⁺ T cells to cell proliferation and cytolytic activity. *J. Exp. Med.* **148**:1523.
14. Shearer, G. 1974. Cell mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* **4**:527.
15. Jung, H., K. Pfizenmaier, A. Starzinski-Powitz, M. Rölinghoff, and H. Wagner. 1978. Primary in vitro sensitization of virus specific cytotoxic T lymphocytes. *Immunology.* **34**:763.
16. Wagner, H., C. Hardt, R. Bartlett, M. Rölinghoff, and K. Pfizenmaier. Intrathymic differentiation of cytotoxic T lymphocyte (CTL) precursors. I. The CTL immunocompetence of PNA⁺ (cortical) and PNA⁻ (medullary) Lyt 123 thymocytes. *J. Immunol.* In press.
17. Reisner, Y., M. Linker-Israeli, and N. Sharon. 1976. Separation of mouse thymocytes into two subpopulations by the use of peanut agglutinin. *Cell. Immunol.* **25**:129.
18. Shen, F. W., J. S. McDougal, J. Bard, and S. P. Cort. 1980. Developmental and communicative interrelations of Lyt123 and Ly1 cell sets. *J. Exp. Med.* **151**:566.
19. Bach, F. H., and B. J. Alter. 1978. Alternative pathways of T lymphocyte activation. *J. Exp. Med.* **148**:829.
20. Melief, C. J., M. Y. van der Meulen, B. J. Christiaans, and P. de Greeve. 1979. Cooperation between subclasses of T lymphocytes in the in vitro generation of cytotoxicity against a mutant H-2K difference. An analysis with anti-Lyt antisera. *Eur. J. Immunol.* **9**:7.
21. Gillis, S., N. A. Union, P. E. Baker, and K. A. Smith. 1979. The in vitro generation and sustained culture of nude mouse cytolytic T-lymphocytes. *J. Exp. Med.* **149**:1460.
22. Wagner, H., C. Hardt, K. Heeg, M. Rölinghoff, and K. Pfizenmaier. 1980. T cell derived helper factors allows in vivo induction of cytotoxic T cells in nu/nu mice. *Nature (Lond.)*. **284**:278.