FUNCTIONAL SUBCLASSES OF T LYMPHOCYTES BEARING DIFFERENT Ly ANTIGENS

I. The Generation of Functionally Distinct T-Cell Subclasses is a Differentiative Process Independent of Antigen*

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T lymphocytes mediate many immunologic functions. For example, they generate cytotoxic responses to alloantigens (1, 2), exert helper (3) and suppressor (4) effects on the production of antibody, and initiate graft-vs.-host responses (5). We do not know whether this diversity of function reflects a functional heterogeneity of T lymphocytes existing before antigen stimulation. If this were the case, then the response of a T-cell clone to stimulation by antigen would be limited to the particular immune function for which it had already been programmed during the differentiation of that T cell. Alternatively, antigen stimulation of a single T cell may induce the formation of progeny that can mediate the complete range of T-dependent responses. These alternatives are illustrated in Fig. 1.

One can pose the question in this way: *is it possible to separate subclasses of T cells from nonimmune animals that are already determined to express, respectively, helper activity or cytotoxic activity before they encounter antigen?* A direct approach to this question could be based upon the use of alloantisera that would define cell surface components expressed selectively on one or another T-cell subclass. Genes coding for such components would most likely be expressed exclusively in T cells.

Because the Ly antigens are said to be reduced on lymphoid cells from neonatally thymectomized mice (6), and have not been detected on the surface of non lymphoid cells (7, 8), they may represent components expressed exclusively on the surface of cells undergoing thymus-dependent differentiation. Each Ly system comprises a genetic locus (*Ly-1* on chromosome 19, *Ly-2* and *Ly-3* closely linked on chromosome 6) each with two alternative alleles, each allele specifying an alternative alloantigen denoted 1 and 2. Thus, the *Ly-1* alleles specify alloantigens Ly-1.1 and Ly-1.2, and all inbred mice express one or the other on their thymocytes; and similarly for *Ly-2 and Ly-3* (9).

* This work was supported in part by National Cancer Institute CA-08748, CA-16889, and National Institute of Allergy and Infectious Diseases grant AI-12184.

Shiku and his colleagues observed that effector killer cells, ie. cells that have already responded to antigen, express a distinctive profile of Ly antigens (10). In the study reported here, we have extended the use of the Ly systems to attack the fundamental question posed above, namely whether or not the generation of functional T-cell diversity precedes the encounter with antigen. We find that subclasses of peripheral T cells with different immunological functions and biological characteristics, distinguishable by expression of different Ly alloantigens, pre-exist in mice that have not been immunized. This indicates that diversification of T-cell function, resulting in cells committed to express either helper or killer activity, is a differentiative process that has taken place *before* T cells meet antigen and that these diverse effector cells are derived from different maturational lines of T cells.

Materials and Methods

Notations. The notation for congenic mice and antisera follows Shiku et al. (10).

Mice. C57BL/6 (B6)[1] (Ly phenotype 1.2,2.2,3.2) and B10.D2 were obtained from the Jackson Laboratories, Bar Harbor, Maine, congenic lines B6/Ly-1.1 (Ly-1.1,2.2,3.2), B6/Ly-2.1 (Ly-1.2,2.1,3.2), and B6/Ly-2.1,Ly-3.1 (Ly-1.2,2.1,3.1) from EAB (ref. 12, Table II); B10.T(6R) and AQR were bred by H. Cantor from breeders supplied by Dr. K. Melief of Tufts Medical School; (B6 × BALB)F_1 (Ly-1.2,2.2,3.2) was bred by H. Cantor.

Antisera. Anti-Ly-1.2, anti-Ly-2.2 and anti-Ly-3.2, and anti-Thy-1.2 are described in Shiku et al. (ref. 10, Table III). The Ly antisera we used, diluted 1:10, were absorbed once with 120 × 10^6 syngeneic thymus + LNC/ml to remove autoantibody.

Complement (C)-Dependent Cytotoxicity Assay. 10–40 × 10^6 cells/ml (^{51}Cr-labeled; 30 min, 100 μCi/ml) were incubated with Ly antiserum diluted in phosphate-buffered saline with 5% fetal calf serum (PBS-FCS) for 1/2 h at 37°C, washed once, brought up in 1 ml of freshly thawed rabbit serum (diluted 1:8 in PBS), and incubated for a further 1/2 h at 37°C. Rabbit sera selected for C were preabsorbed with mouse cells in the presence of EDTA (see 10), which reduced background cytotoxicity for spleen and LNC, under the conditions described above, to <12%.

Sequential Lysis with Different Ly Antisera and C. The proportions of cells displaying one or more Ly antigens were estimated from the lytic effects resulting from sequential exposure to two different Ly antisera; the protocol using B6 LNC as target cells is illustrated (with controls) as follows:

Step 1 (+ C)	Cell lysis*	Step 2§ (+ C)	Lysis of remaining population
	%		%
(a) Anti-Ly-2.2	33	Anti-Ly-1.2	21
(b) NMS	Standard‡	Anti-Ly-1.2	51
(c) Anti-Ly-1.2	52	Anti-Ly-1.2	0
(d) Anti-Ly-2.2	33	Anti-Ly-2.2	0
(e) NMS	Standard‡	NMS	Standard‡

* $\frac{\text{cpm (antiserum)} - \text{cpm (NMS)}}{\text{cpm (freeze-thaw)} - \text{cpm (NMS)}} \times 100.$

‡ $\frac{\text{cpm (NMS)}}{\text{cpm (freeze-thaw)}} \times 100$ (= 9% in both Steps 1 and 2 in this particular test).

§ Cells from Step 1 were spun down and resuspended in fresh PBS-FCS before Step 2.

[1] *Abbreviations used in this paper:* BALB, BALB/c; B6, C57BL/6; C, complement; LNC, lymph node cells; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; MLC, mixed lymphocyte culture; NMS, normal mouse serum; PBS-FCS, phosphate-buffered saline + 5% fetal calf serum; PFC, plaque-forming cells.

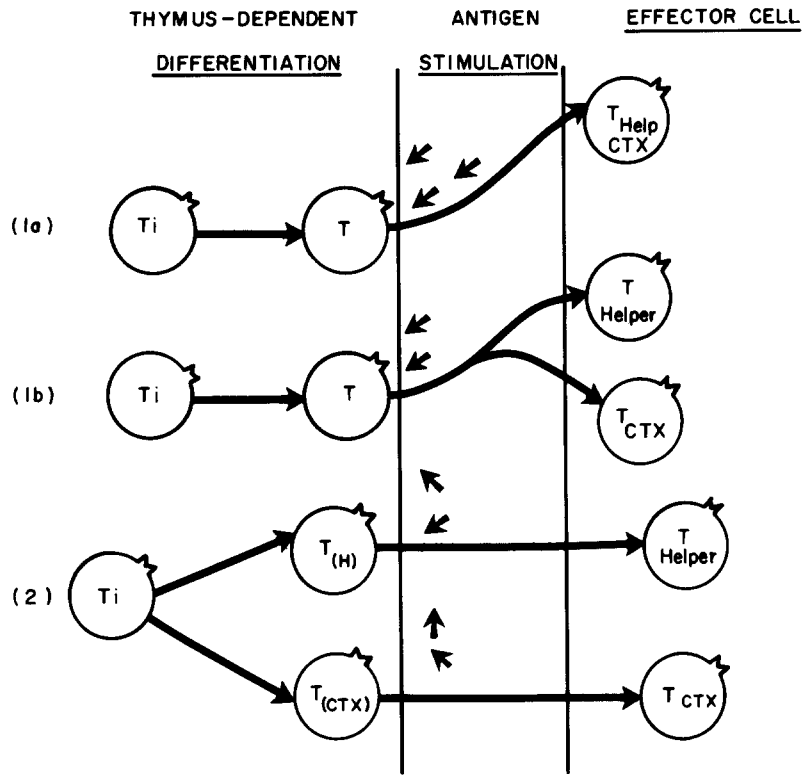

FIG. 1. Alternative maturation schemes to account for diversity of T-cell function. (1a) After stimulation by antigen, a single T cell mediates all T-cell function. (1b) After stimulation by antigen, a single T cell generates functionally different progeny. (2) Subclasses of T cells are programmed for different T-cell functions during differentiation *before* contact with antigen. (T_i = immature T cell).

This analysis is valid because maximum killing with each Ly antiserum is obtained in one incubation, see (*c*) and (*d*) above, and because pretreatment with NMS + C does not alter susceptibility to subsequent lysis with anti-Ly + C, compare (*b*) Step 2 with (*c*) Step 1. Therefore, all such tests included controls (*b*) to (*e*).

Use of Ly Congenic Mice for Confirming Specificity of Ly Antisera. The specificity of any effects of a given Ly antiserum + C upon the immunologic function of B6 cells was confirmed by examining the effects of the same antiserum on cells from B6 congenic mice which are genetically identical to B6 except for the Ly locus in question (negative controls) as follows: (*a*) anti-Ly-1.2 tested on B6/Ly-1.1 cells, (*b*) anti-Ly-2.2 on B6/Ly-2.1, and (*c*) anti-Ly-3.2 on B6/Ly-2.1,3.1. If there is no effect upon the immune function of these control cells, then the effect of the Ly antiserum on B6 cells must be specific.

Purification of T Lymphocytes. Purification of T cells by elution from Sephadex G200 columns coated with rabbit antimouse immunoglobulin (MIg) according to Schlossman and Hudson (11) permitted virtually 100% T-cell recovery from spleen or LNC; contamination by Ig^+ cells was approximately

2–5%. In some experiments, T cells were enriched using nylon wool columns according to Julius et al. (12), resulting in a recovery of 60–80% of Thy-1$^+$ cells, and a 5–15% contamination by Ig$^+$ cells; despite this incomplete T-cell recovery, cells purified by nylon wool and rabbit anti-MIg columns had similar functional properties.

Assays of Immunologic Function. (*a*) Sensitization of T lymphocytes and measurement of cytotoxic activity in a 4-h ^{51}Cr release assay is described elsewhere (2). Briefly, 5×10^5 responder cells and 3×10^5 irradiated (2,500 R) BALB or (B6 × BALB)F_1 stimulator cells were incubated at 37°C in Falcon 3040 plates for 5 days in 7% CO_2. Cytotoxic activity for each sensitized cell population was calculated thus:

$$\% \text{ Lysis} = \frac{\text{cpm (sensitized cells)} - \text{cpm (unsensitized cells)}}{\text{cpm (freeze-thaw)}} \times 100.$$

(*b*) The ability of cell populations to generate cytotoxicity in vivo was determined by injecting parental (B6) cells (*H-2^b/H-2^b*) into irradiated (900 R) (B6 × BALB)F_1 recipients (*H-2^b/H-2^d*) aged 4–6 wk. 4 days later anti-*H-2^d* cytotoxic activity in spleen was measured against ^{51}Cr-labeled LSTRA target cells in a 4-h assay (2). (*c*) In other experiments, proliferative responses of allogeneic mixed cell cultures were measured by incorporation of [^{3}H]T 72–96 h after initiation. These cultures contained 10% human sera and were pulsed with [^{3}H]T according to Nabholz et al. (13). (*d*) Helper activity after treatment with Ly antisera + C was assessed as follows: cell populations passed through rabbit anti-MIg or nylon columns were treated with the different Ly antisera + C and inoculated intravenously into syngeneic irradiated hosts (15×10^6 viable cells/mouse); this inoculum was combined with 10^7 viable spleen cells (pretreated with anti-Thy-1 + C, as a source of B cells). Recipient spleens were tested for anti-SRBC PFC 6 days later.

In both the in vitro and in vivo test systems described above, the concentration of viable cells remaining after pretreatment with antiserum or NMS + C was equalized for all groups.

Results

I. Expression of Ly Antigens on Thymocytes and Peripheral T Cells as Indicated by the C-Dependent Cytotoxicity Assay. Each Ly antiserum (Ly-1.2,Ly-2.2, or Ly-3.2) lysed about 90% of B6 thymocytes, but only 35-60% of cortisone-resistant thymocytes (Fig. 2). One explanation is that although the majority of thymocytes [corresponding to the TL$^+$ phase population (9)] display all three Ly antigens, many cortisone-resistant thymocytes do not. Anti-Thy-1.2 lysed approximately 40% of spleen cells, Ly-1 antiserum 30%, Ly-2 or Ly-3 antiserum 15-20%, and all three Ly antisera together around 35% (Fig. 3 A). The respective figures for spleen suspensions enriched for T cells by passage through a column coated with rabbit anti-MIg were 85% (Thy-1), 65% (Ly-1), 35-45% (Ly-2 or Ly-3), and 80% (Ly-1,2 and 3) (Fig. 3 B).

These data: (*a*) show that the relative proportions of cells expressing different Ly antigens in spleen are similar to those of the cortisone-resistant thymocyte

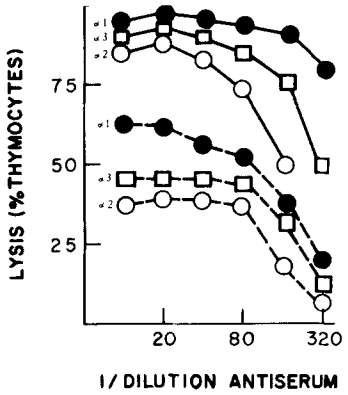

FIG. 2. Complement-dependent lysis of thymocytes by Ly antisera. Lysis of thymocytes from untreated B6 mice (—) and from B6 mice treated with cortisone (2.5 mg cortisone acetate i.p. 48 h previously) (---), by anti-Ly-1.2 (●), anti-Ly-2.2 (○) or anti-Ly-3.2 (□). (For calculations of cells lysed %, see Materials and Methods)

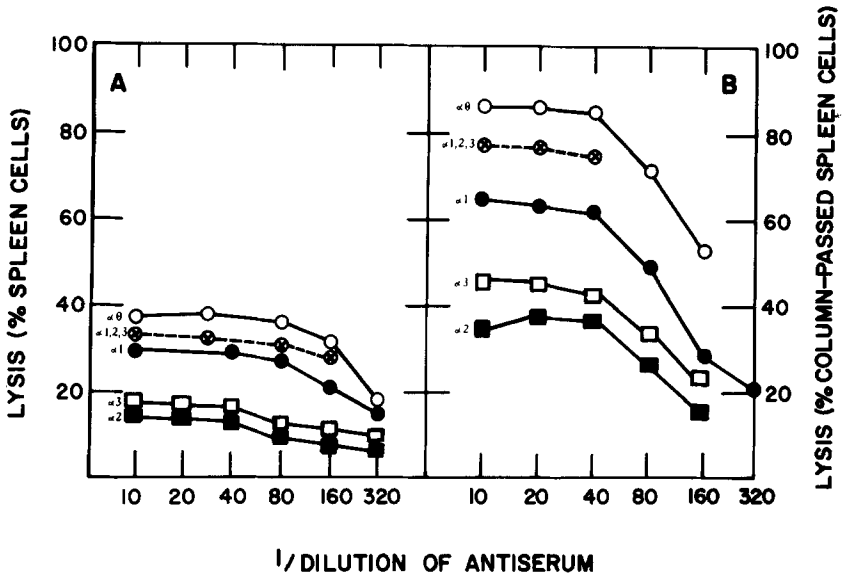

FIG. 3. Complement-dependent lysis of spleen cells by Ly antisera. Lytic activity of anti Thy-1.2 (○), anti Ly-1.2 (●), anti Ly-2.2 (■) and anti Ly-3.2 (□) against pooled B6 spleen cells (A) and B6 spleen cells passed through a rabbit anti-MIg coated column (B). Lysis by all 3 Ly antisera combined is also shown (⊗) (starting dilution of 1/10 for each of the three Ly antisera in the mixture).

population (Fig. 2), and (*b*) imply that the Ly antisera define subpopulations of Thy-1$^+$ lymphocytes in peripheral lymphoid tissues.

II. Estimation of the Proportions of Spleen and LNC Marked by Different Ly Antigens. To estimate the proportions of peripheral cells that displayed different combinations of Ly antigens, the lytic activity of each Ly antiserum was assessed against populations of spleen and LNC that had been pretreated with either NMS or another Ly antiserum in the presence of C (see Materials and

Methods). The results (Table I) indicate that approximately 30% of Thy-1$^+$ cells in peripheral lymphoid tissues display predominantly Ly-1, approximately 7% Ly-23, and approximately 50% all three Ly antigens. These experiments also show that cells resistant to anti-Thy-1.2 serum are not susceptible to subsequent exposure to anti Ly sera; *i.e.*, Ly expression is confined to Thy-1$^+$ cells.

III. Ontogeny of Subclasses of Ly-Bearing Cells in Peripheral Lymphoid Tissues, and their Dependence on the Thymus in Adult Life. To study the ontogeny of the three subclasses of Ly$^+$ cells defined above (Ly-123$^+$, Ly-1$^+$, Ly-23$^+$), spleen cells from mice of different ages were examined by sequential lysis with different Ly antisera. Virtually all *Ly*$^+$ spleen cells 1 wk after birth were Ly-123$^+$ (Fig. 4). Ly-1$^+$ and Ly-23$^+$ cells, although undetectable in neonatal life,

TABLE I

*Proportions of Spleen and LN Lymphocytes Bearing One or More Ly Antigens, Calculated from Results of Sequential Lysis with Different Ly Antisera**

Ly phenotypes inferred	% of total spleen + LN population		
	Exp. 1	Exp. 2	Exp. 3
For Thy-1$^-$ Cells:			
Ly$^+$	0	1	0
	% of Thy-1$^+$ population		
For Thy-1$^+$ cells:			
Ly-1$^+$2$^+$3$^+$	51	56	49
Ly-1$^+$	32	30	33
Ly-2$^+$3$^+$	6	8	7
Ly-2$^+$	0	0	0
Ly-3$^+$	0	2	1

* See Materials and Methods for details of procedure.

gradually increased in numbers so that by the 10th week of life they together accounted for roughly 20% of the total spleen cell population.

The effect of removing the thymus in adult life upon the concentration of these Ly subclasses in the spleen was then examined. Spleen cells obtained 3 wk after thymectomy or sham thymectomy (performed on 7-wk old mice) were sequentially treated with Ly antisera. These experiments indicate that adult thymectomy resulted in approximately a 50% decrease in the proportion of Ly-123$^+$ cells and a slight increase in the relative proportions of Ly-1$^+$ and Ly-23$^+$ cells as compared with sham-operated controls (Fig. 5).

IV. The Effect of Pretreatment with Ly Antisera and C upon the Capacity of Lymphoid Cells to Develop Helper and Cytotoxic Activities in Irradiated Hosts. Nylon-column-passed spleen and LNC (pooled) from B6 mice were treated with Ly antisera + C and resuspended to give a standard concentration of viable cells. Half the cells from each group were combined with anti-Thy-

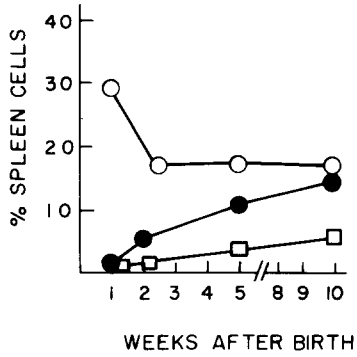

FIG. 4. Ontogeny of subclasses of Ly$^+$ cells in spleen. Proportions of Ly-123$^+$ (○), Ly-1$^+$ (●) and Ly-23$^+$ (□) cells at various times after birth, calculated by sequential lysis with the different Ly antisera + C (see Materials and Methods, and Table I).

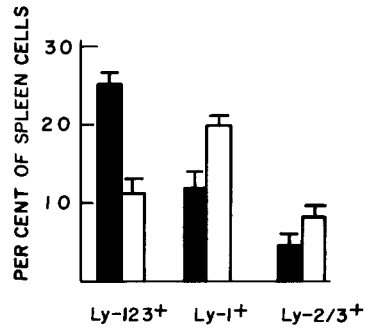

FIG. 5. Effect of adult thymectomy on different subclasses of Ly$^+$ lymphocytes. Proportions of Ly-123$^+$, Ly-1$^+$, and Ly-23$^+$ cells in spleen of thymectomized (□) and sham-operated (■) mice. (Spleen cells taken from 10-wk old B6 mice thymectomized, or sham-operated, 3 wk previously.)

1.2-treated B6 spleen cells (source of B cells) and inoculated into B6 (800 R) recipients together with 7×10^6 SRBC. The other half was inoculated i.v. into F_1 hybrid (B6 × BALB) irradiated recipients. After 5 days, spleen cells from the former were assessed for anti-SRBC PFC, and spleen cells from the F_1 recipients for anti *H-2*^{d} cytotoxic activity. Removal of Ly-1$^+$ cells abolished the subsequent development of PFC activity in irradiated hosts (Table II) but had little effect upon the generation of cytotoxic cells (Fig. 6). By contrast, removal of Ly-2$^+$ or Ly-3$^+$ cells almost eliminated the generation of cytotoxic cells (Fig. 6) but did not reduce the PFC response (Table II). In control tests, treatment of T cells from congenic B6/Ly-1.1 mice (see Materials and Methods) with anti-Ly-1.2 serum, or cells from B6/Ly-2.1 mice with anti-Ly-2.2 had no effect upon subsequent immune function, indicating the specificity of the results observed for the *Ly* systems named.

V. The Effect of Pretreatment with Ly Antiserum Upon the Generation of Cytotoxic Activity by Lymph Node Cells In Vitro. To analyze further the cellular basis of the production of cytotoxic activity, T cells were sensitized to alloantigens in vitro. B6 cells treated with anti-Ly-2.2 or anti-Ly-3.2 (and thus

enriched for Ly-1$^+$ T cells) were unable to generate appreciable cytotoxic activity as a result of sensitization in vitro (Fig. 7), thus confirming the effects of these antisera in the circumstances of sensitization in vivo (Section IV above). Pretreatment with anti-Ly-1.2, thus enriching for Ly-23$^+$ cells, produced an

TABLE II

The Effect of Pretreatment with Ly Antiserum and C Upon the Capacity of B6 Lymphoid Cells to Develop Helper Function in a Primary Antibody Response In Vivo

B6 cells treated with:	B cells ($\times 10^7$)*	PFC/spleen (direct/developed)	
		Mean	Range
NMS	+	1,450/2,160	866-2,450/950-2,840
[No cells]	+	11/0	0-30/0
Anti-Thy-1.2	+	42/10	0-95/0-20
Anti-Ly-1.2	+	188/35	20-305/0-65
Anti-Ly-2.2	+	1,890/2,940	1,050-3,920/2,400-3,960
Anti-Ly-3.2	+	1,850/2,360	1,260-2,940/1,960-2,760
NMS	−	25/0	—
Anti-Ly-1.2 (vs B6/Ly-1.1 cells = Ly specificity control)	+	1,050/1,450‡	—

* Obtained by treatment of B6 spleen cells with anti-Thy-1.2 + C.

‡ This control was performed in 2 of the 5 experiments presented, and did not differ significantly from NMS-treated B6 cells in the same experiments.

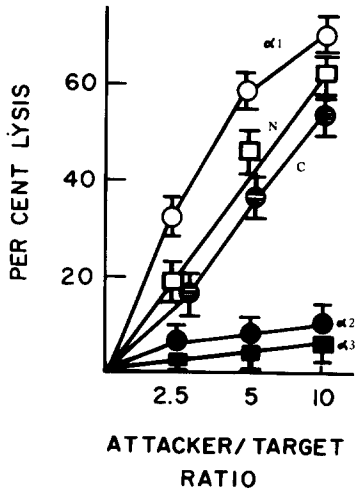

FIG. 6. Effect of treatment with Ly antiserum and C upon the capacity of parental LN cells to generate killer activity in F_1 hybrid recipients. After treatment of B6 LN cells with either NMS or each of the Ly antisera, equal numbers of viable cells were injected intravenously into (B6 × BALB)F_1 irradiated (900 R) hosts were harvested and assayed for killer activity against LSTRA (H-2^d) target cells. The effects of pretreating the B6 donor cells, before in vivo sensitization, with: anti-Ly-1.2 (○), anti-Ly-2.2 (●), anti-Ly-3.2 (■) or NMS (□) are shown. The effect of anti-Ly-2.2 on the ability of cells from B6/Ly-2.1 donors, to generate a cytotoxic response is also shown as a specificity control (⊜) (See Materials and Methods for details). Vertical bars denote the limits of one standard error, based upon triplicate cytotoxic measurements.

increase in the generation of cytotoxic activity by comparison with: (*a*) the activity generated by B6 cells pretreated with NMS, or (*b*) the activity of B6/Ly-1.1 congenic cells pretreated with anti-Ly-1.2 (Ly specificity control). To determine whether the Ly-23$^+$ phenotype of the precursor of the killer cell (as demonstrated by these experiments) is retained by the killer cell which is generated from it, we next examined the effects of treating with Ly antiserum

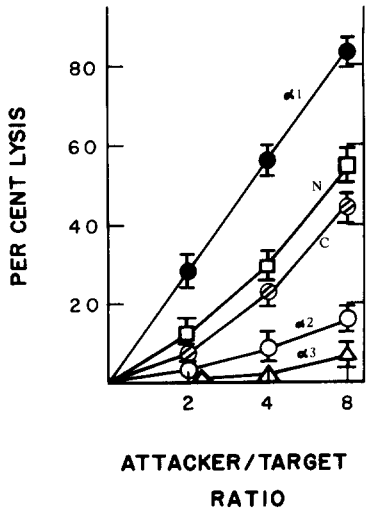

FIG. 7. Effect of pretreatment with Ly antisera upon the capacity of T cells to generate cytotoxic activity to alloantigens in vitro. Nylon passed lymph node cells from B6 mice were treated with Ly antisera, or NMS; equal numbers of remaining viable cells were sensitized to irradiated BALB cells for 5 days in vitro. Cytotoxic responses produced at the end of this sensitization period by cells that had been pretreated with anti-Ly-1.2 (●), anti-Ly-2.2 (○), anti-Ly-3.2 (△) and NMS (□) are shown. The cytotoxic response generated by B6/Ly-2.1 cells treated with anti-Ly-2.2 (Ly specificity control) is also indicated (⊜). Although not shown, pretreatment of lymph node cells with anti-Thy-1.2 + C abolished the ability of these cells to generate a cytotoxic response. Vertical bars denote the limits of one standard error, based upon triplicate cytotoxic measurements.

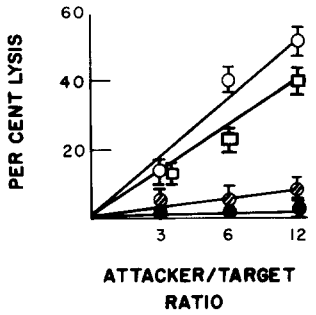

FIG. 8. The effect of Ly antisera + C on killer cell activity. B6 cells that had been sensitized in vitro to irradiated BALB cells were treated with Ly antisera + C, adjusted to equal numbers of viable cells and tested for lytic activity against ^{51}Cr-labeled LSTRA cells. The lytic activity of cells after treatment with anti-Ly-1.2 (○), anti-Ly-2.2 (⊜), anti-Ly-3.2 (●), and NMS + C (□) is shown. Vertical bars denote limits of one standard error.

after sensitization of B6 cells in vitro (i.e., treatment of preformed killer cells) (Fig. 8). Between 70 and 90% of cytotoxic activity was eliminated by anti-Ly-2.2 or anti-Ly-3.2; none by anti-Ly-1.2. Thus, the Ly phenotype of the prekiller cell is stable, and also distinguishes its killer progeny.

VI. The Effects of Pretreatment with Ly Antiserum on the Proliferative Response of Lymphoid Cells to Allogeneic Lymphocytes (Table III). The findings above signify that cytotoxic effector cells originate from Ly-23$^+$ T cells, but do not indicate the relative contributions of the different Ly$^+$ subclasses to the proliferative or recognition phase of the response to alloantigens. For this

TABLE III
Recognition of Different Alloantigens by Subpopulations of T Cells Distinguished by Different Ly Antigens

Antiserum pretreatment*	Incompatibility‡					
	H-2 + non-*H-2*		*H-2*		*I* + *S*	
	E-B	E/B	E-B	E/B	E-B	E/B
NMS	8249	13.31	5310	8.10	6936	10.97
Anti Thy-1.2	331	1.10	321	1.34	129	1.07
Anti Ly-1.2	4931	6.79	3960	4.41	2424	3.08
Anti Ly-2.2	5344	6.88	3485	3.60	11591	12.06
Anti Ly-3.2	5150	6.91	4360	4.95	12116	13.81

	Percent reduction in MLC activity§		
Anti Thy-1.2	96	94	98
Anti Ly-1.2	40	25	65
Anti Ly-2.2	35	34	0 (+67)
Anti Ly-3.2	38	18	0 (+77)

* Following exposure to antiserum + C, cells in each group were adjusted to the same final concentration of viable cells.

‡ *H-2* + non-*H-2* = B10 + BALB (2,500 R); *H-2* = B10+ B10.D2 (2,500 R); I + S = B10.T(6R) + AQR (2,500 R); E (experimental) = cpm (responder + stimulator: 2,500 R); B (background) = cpm (responder + responder: 2,500 R).

§ Percent reduction in MLC activity = $\frac{\text{E-B (anti-Ly + C)}}{\text{E-B (NMS + C)}}$.

purpose, we assessed the effects of pretreatment with Ly antisera upon MLC responses. Pretreatment of responder cells with each Ly antiserum reduced subsequent proliferation in MLC by about 50%, suggesting that although Ly-1$^+$ cells did not directly contribute to the production of the cytotoxic response (Section V above), they nonetheless constitute a substantial proportion of the cells that are stimulated to proliferate by alloantigens.

To investigate the possibility that Ly-1$^+$ cells were responding to alloantigens controlled by genes outside the major histocompatibility complex (MHC), which

do not normally elicit a cytotoxic response, we tested the effect of pretreatment with Ly antisera upon the MLC response of B10 cells stimulated with irradiated B10.D2 congenic cells. Again pretreatment with anti-Ly-1.2 reduced the MLC response by about 50-60% and so did anti-Ly-2.2 and anti-Ly-3.2. Thus, both Ly-1$^+$ and Ly-23$^+$ cells contribute to the MLC response to antigens of the MHC. Finally, the participation of Ly subclasses in the proliferative response to antigens coded by the $I + S$ region of the MHC is indicated in Table III. Here we tested the effects of pretreatment with Ly antisera upon the MLC response of B10.T(6R) cells stimulated with irradiated AQR cells. These data show that Ly-1$^+$ T cells accounted for almost all the proliferative response to I region ($+ S$) determinants; in fact, pretreatment with anti-Ly-2 or anti-Ly-3 resulted in substantially increased proliferative responses to these antigens.

Discussion

Antigen-stimulated T cells have various immune functions, including secretion of pharmacologic mediators influencing cellular responses (14), amplification or suppression of antibody responses (3, 4), and generation of cells capable of specifically destroying target cells of contact. There is increasing evidence that, after antigenic stimulation has occurred, these individual functions may be performed by T lymphocytes that have different physical and biologic properties (15, 16), and are distinguished by different *Ly* markers (10). But there has so far been no direct evidence as to whether the cells of a single clone develop diverse functions after contact with antigen (Fig. 1; Model 1B) or whether T-cell differentiation involves the evolution of subclasses of functionally different T cells, independently of triggering by antigen (Fig. 1; Model 2).

This is a question of fundamental relevance to the physiology of the immune system, because it involves a choice between, on the one hand, a true differentiative process for which a corresponding regulatory mechanism must be proposed and sought, and on the other hand, a quite different mechanism that would depend on fortuitous antigenic stimulation and so would be excluded from the category of physiological internally regulated differentiative events.

According to our data, for at least two T-cell functions, generation of killer activity and helper activity, respectively, functional commitment is manifest before contact with antigen: (*a*) According to the ^{51}Cr release cytotoxicity assay with Ly antisera, only 50% of Thy-1$^+$ spleen cells are Ly-123$^+$, the rest being Ly-1$^+$ or Ly-23$^+$; (*b*) Lymphoid populations depleted of Ly-1$^+$ T cells (and thus enriched for Ly-23$^+$ cells) are unable to generate appreciable helper responses but their capacity to generate cytotoxic activity in vivo and in vitro is unimpaired; (*c*) Lymphoid cell populations depleted of Ly-23$^+$ cells (and consequently enriched for Ly-1$^+$ cells) produced substantial helper responses but their ability to generate cytotoxic activity in vitro and in vivo was greatly reduced.

Thus, T cells have already been instructed to express exclusively helper function or cytotoxic function *before* they encounter antigen. We have not investigated whether the different Ly components on the T-cell surface contribute directly to the expression of these functions, though Shiku et al. give evidence against this as far as cytotoxic effector function is concerned (10), or whether they perform some other function peculiar to cells that are destined to undertake these activities upon contact with antigen.

In MLC, the Ly-23$^+$ T-cell subclass that gives rise to killer cells responds to MHC antigens coded by the *H-2K* and *H-2D* regions, whereas the MLC response to *I*-region antigens is confined to the Ly-1$^+$ (helper) subclass. This is consistent with reports indicating that cytotoxicity can be generated in response to *H-2K* and *H-2D* region antigens (13), and although total lytic activity may be augmented by additional *I*-region differences (17) the latter are not thought to suffice for triggering cytotoxicity in the systems currently used, and probably do not contribute to the specificity of cytotoxic effector cells (17).

Recognition of *I*-region products (including Ia) by the Ly-1$^+$ helper subclass is also relevant to evidence that these determinants: (*a*) are found mainly on B cells (18), (*b*) are closely associated with or identical to B-cell Fc receptors (19), and (*c*) may play a role in T-B interactions (20). Possibly the proliferative response of Ly-1$^+$ helper cells to Ia antigens on allogeneic B cells may pre-empt their capacity to exert helper activity (20). Recognition of antigen associated with syngeneic Ia molecules on the surface of B cells or macrophages may by contrast favor expression of helper function.

Our data imply that TL$^+$Ly-123$^+$ cells generate three (TL$^-$) subclasses of T cells, denoted Ly-1$^+$, Ly-23$^+$ and Ly-123$^+$, as a normal differentiative process that is independent of exposure to antigen. Whether the Ly-1$^+$ and Ly-23$^+$ subclasses are generated from intermediary TL$^-$Ly-123$^+$ precursors has yet to be decided. Possibly relevant to this question are the findings that: (*a*) all Thy-1$^+$ cells in peripheral tissues in the 1st week of life are Ly-123$^+$, but the proportion of this cell type declines with time in favor of Ly-1$^+$ and Ly-23$^+$ cells, and (*b*) the selective reduction of Ly-123$^+$ cells seen shortly after adult thymectomy may suggest that these cells are transitional peripheral derivatives of TL$^+$Ly-123$^+$ thymocytes. If the Ly-123$^+$ subclass is transitional, the further differentiation of any antigen-specific clone to yield Ly-1$^+$ or Ly-23$^+$ progeny may be regulated by genes within the MHC, i.e., *Ir* genes. Alternatively, the Ly-123$^+$ subclass may be a separately differentiated regulatory population, perhaps capable of exerting immunosuppressive effects after stimulation with antigen. In this latter case, the relative proportions of Ly-123$^+$ and Ly-1$^+$ cells with specificity for a given antigen would determine the net helper effect to that antigen. Isolation of Ly-123$^+$ cells from peripheral tissues may yield definitive evidence for either of these developmental pathways and should permit functional studies of this T-cell subclass.

We have shown that both the prekiller cell and killer cell express the Ly-23 phenotype. Whether Ly-1$^+$ cells, which also recognize certain alloantigens according to the MLC criterion, influence the generation of Ly-23$^+$ killer cells, is dealt with in the following report (21).

Summary

Ly alloantigens coded by two unlinked genetic loci (*Ly-1* and *Ly-2/Ly-3*) are expressed on lymphoid cells undergoing thymus-dependent differentiation. Peripheral Thy-1$^+$ cells from C57BL/6 mice can be divided into three subclasses on the basis of differential expression of Ly-1, Ly-2, and Ly-3; about 50% express all three Ly antigens (Ly-123$^+$), about 33% only Ly-1 (Ly-1$^+$), and about 6–8% Ly-2 and Ly-3 (Ly-23$^+$). Cells of the Ly-123$^+$ subclass are the first peripheral Thy-1$^+$ cells to appear in ontogeny, and are reduced in the periphery shortly after

adult thymectomy. In contrast, Ly-1$^+$ and Ly-23$^+$ subclasses appear later in the peripheral tissues than do Ly-123$^+$ cells, and are resistant to the early effects of adult thymectomy.

Peripheral lymphoid populations depleted of Ly-1$^+$ cells and Ly-123$^+$ cells (and thereby enriched for Ly-23$^+$ cells) were incapable of developing significant helper activity to SRBC but generated substantial levels of cytotoxic activity to allogeneic target cells. The same lymphoid populations, depleted of Ly-23$^+$ cells and Ly-123$^+$ cells (and thereby enriched for Ly-1$^+$ cells), produced substantial helper responses but were unable to generate appreciable levels of killer activity.

These experiments imply that commitment of T cells to participate exclusively in either helper or cytotoxic function is a differentiative process that takes place before they encounter antigen, and is accompanied by exclusion of different Ly groups, Ly-23 or Ly-1 respectively, from TL$^+$Ly-123$^+$ T-cell precursors. It is yet to be decided whether the TL$^-$ phase Ly-123$^+$ subclass is a transitional form or a separately differentiated subclass with a discrete immunologic function.

We are grateful for the excellent technical assistance of Ms. Joan Hugenberger and Ms. Linda Gassett, and for the assistance of Ms. Martha Mann and Mr. Austin Segel.

Received for publication 18 December 1974.

References

1. Goldstein, P., H. Wigzell, H. Blomgren, and E. A. J. Svedmeyer. 1974. Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of T lymphocytes for the killing of allogeneic target cells. *J. Exp. Med.* **135**:890.
2. Cantor, H., E. Simpson, V. Sato, G. Fathman, and L. A. Herzenberg. 1975. Characterization of subpopulations of T lymphocytes. Separation and functional studies of peripheral T cells binding different amounts of fluorescent anti Thy-1.2 antibody. *Cell. Immunol.* **15**:180.
3. Transplantation Reviews, Vol. 1. 1969. G. Möller, editor. Williams and Wilkins Co., Baltimore, Md.
4. Gershon, R. K. 1974. T cell suppression. *Contemp. Top. Immunobiol.* **3**:1.
5. Cantor, H. 1972. The effects of anti-theta serum upon graft vs. host activity of spleen and lymph node cells. *Cell. Immunol.* **3**:461.
6. Schlesinger, M. 1972. Antigens of the thymus. *Prog. Allergy.* **16**:214.
7. Boyse, E. A., M. Miyazawa, T. Aoki, and L. J. Old. 1968. Ly-A and Ly-B: two systems of lymphocyte isoantigens in the mouse. *Proc. Roy. Soc. Lond. B. Biol. Sci.* **170**:175.
8. Boyse, E. A., K. Itakura, E. Stockert, C. Iritani, and M. Miura. 1971. Ly-C: a third locus specifying alloantigens expressed only on thymocytes and lymphocytes. *Transplantation.* **11**:351.
9. Itakura, K., J. J. Hutton, E. A. Boyse, and L. J. Old. 1972. Genetic linkage relationships of loci specifying differentiation alloantigens in the mouse. *Transplantation.* **13**:239.
10. Shiku, H., P. Kisielow, M. A. Bean, T. Takahashi, E. A. Boyse, H. F. Oettgen, and L. J. Old. 1975. Expression of T-cell differentiation antigens on effector cells in cell-mediated cytotoxicity in vitro: evidence for functional heterogeneity related to surface phenotype of T cells. *J. Exp. Med.* **141**:227.
11. Schlossman, S. F., and L. Hudson. 1973. Specific purification of lymphocyte populations on a digestible immunoabsorbant. *J. Immunol.* **110**:313.

12. Julius, M., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol.* **112**:420.
13. Nabholz, I., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rijnbeek, and D. C. Shreffler. 1974. Cell mediated cell lysis in vitro: Genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* **4**:378.
14. David, J. R., and R. A. David. 1972. Cellular hypersensitivity and immunity. Inhibition of macrophage migration and the lymphocyte mediators. *Prog. Allergy* **16**:300.
15. Dennert, G. 1974. Evidence for non-identity of T killer and T helper cells sensitized to allogeneic cells. *Nature (Lond.)*. **249**:358.
16. Tigelaar, R. E., and R. M. Gorczynski. 1974. Separable populations of activated thymus-derived lymphocytes identified in two assays for cell-mediated immunity to murine tumor allografts. *J. Exp. Med.* **140**:267.
17. Schendel, D. J., B. J. Alter, and F. H. Bach. 1973. Involvement of LD and SD region differences in MLC and CML in a 3 cell experiment. *Transplant. Proc.* **5**:1651.
18. Shreffler, D. C., and D. S. Chella. 1975. The H-2 major histocompatability complex and the *I* immune response region: genetic variation, function and organization. *Adv. Immunol.* In press.
19. Dickler, H. B., and D. H. Sachs. 1974. Evidence for identity or close association of the Fc receptors of B lymphocytes and alloantigens determined by the Ir region of the *H-2* complex. *J. Exp. Med.* **140**:779.
20. Katz, D. H., M. Graves, M. E. Dorf, H. Dimuzio, and B. Benacerraf. 1975. Cell interactions between histoincompatible T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J. Exp. Med.* **141**:263.
21. Cantor, H., and Boyse, E. A. 1975. Functional subclasses of T lymphocytes bearing Different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. *J. Exp. Med.* **141**:1390.