FUNCTIONAL SUBCLASSES OF T LYMPHOCYTES BEARING DIFFERENT Ly ANTIGENS

II. Cooperation Between Subclasses of Ly$^+$ Cells in the Generation of Killer Activity*

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Peripheral T cells can be subclassified on the basis of differential expression of cell surface antigens belonging to three *Ly* systems, *Ly-1*, *Ly-2*, and *Ly-3* (1). One subclass, bearing all three of these Ly determinants (Ly-123$^+$), appears early in neonatal life and is selectively depleted shortly after adult thymectomy. Two other subclasses, Ly-1$^+$ and Ly-23$^+$, develop later in life and are unaffected shortly after adult thymectomy.

Although both Ly-1$^+$ and Ly-23$^+$ subclasses recognize alloantigens of the major histocompatibility complex (MHC),[1] according to the criterion of thymidine incorporation in the mixed lymphocyte culture (MLC) test, only the Ly-23$^+$ subclass develops killer activity (1).

We are concerned in this report with whether T-cell subclasses other than Ly-23 are indirectly involved in the genesis of killer cells, and we shall show: (*a*) that the development of killer activity by Ly-23$^+$ T cells is amplified by Ly-1$^+$ T cells [which we know provide helper activity during primary antibody responses (1)], and (*b*) that the amplifying Ly-1$^+$ T cells probably recognize alloantigens different from those recognized by the Ly-23$^+$ prekiller cells.

Materials and Methods

Mice. The Ly congenic mouse strains B6/Ly-1.1, B6/Ly-2.1, and B6/Ly-2.1,3.1 are described in previous reports (1, 2).

Antisera. The Ly antisera, anti-Ly-1.2, anti-Ly-2.2, and anti-Ly-3.2 are described previously (2). B10.A anti B10.D2 antiserum ("anti-Beta"), produced by immunization with lymphoid cells (3), recognizes determinants coded by the *Ir-IA* subregion of the MHC (D. Sachs, personal communication).

Complement-Mediated Cytolysis by Ly Antisera. This was performed as described previously (1), except that in one experiment (Results, II), designed to assess the contribution of different Ly$^+$ subclasses to the generation of cytotoxic activity, the concentration of viable cells was not restored after treatment with Ly antiserum + complement (C).

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[1] *Abbreviations used in this paper:* BALB, BALB/c; B6, C57BL/6; C, complement; LN-T, column-purified T cells from lymph nodes; LNC, lymph node cells; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; MLC, mixed lymphocyte culture; NMS, normal mouse serum.

Mixed Lymphocyte Cultures and Assay for Cytotoxic Activity Generated In Vitro. These procedures are described in detail elsewhere (1). In some experiments, total lytic activity produced after MLC sensitization was calculated according to the following formula:

$$\text{Total lytic activity} = \frac{\%\ \text{lysis} \times \text{cell yield/culture}}{5 \times 10^5}$$

To assess Ly expression on killer cells (Figs. 2, 3; Table I) we determined the percent reduction of killer activity after treatment of sensitized cells with Ly antisera + C. The treated cells were washed 2 times and resuspended (10^6 viable cells/ml) before the addition of 10^5 ^{51}Cr-labeled target cells. Percent reduction =

$$1 - \frac{\%\ \text{Lysis (antiserum-treated cells)}}{\%\ \text{Lysis (NMS-treated cells)}} \times 100.$$

Results

I. Dose Response Analysis of Killer Activity Generated by Different Subclasses of Ly$^+$ Cells (Fig. 1). The capacity of different Ly$^+$ subpopulations to generate killer activity was quantitated as follows: B6 column-purified T cells from lymph nodes (LN-T) cells were first treated with different Ly antisera + C. Graded numbers of remaining viable cells were sensitized to a constant number of irradiated BALB cells in vitro. Depletion of Ly-1$^+$ and Ly-123$^+$ cells (by treatment with anti-Ly-1.2), giving four- to fivefold enrichment of Ly-23$^+$ cells after equalizing the viable cell count to that of the normal mouse serum (NMS) + C control, increased killer activity by a factor of 1.5–2 as compared with NMS controls.

Two points are noteworthy regarding this increased killer response after

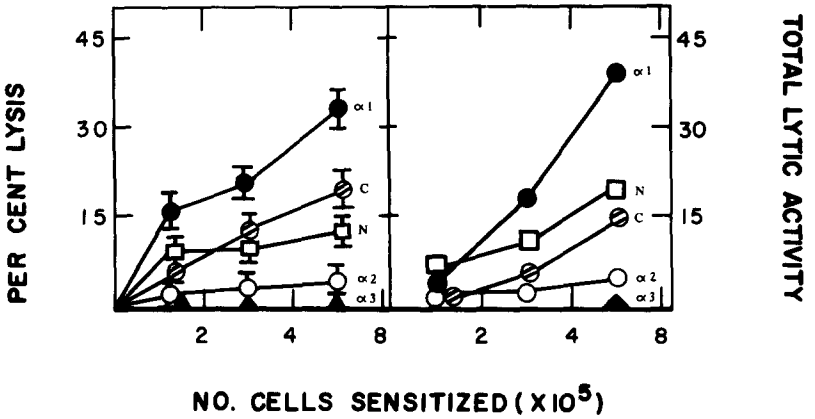

FIG. 1. Dose response analysis of killer activity production by different Ly$^+$ subclasses. After treatment of LN-T lymphocytes with the various Ly antisera [anti-Ly-1 (●), anti-Ly-2 (○), anti-Ly-3 (▲), NMS (□), anti-Ly-2 vs. B6/Ly-2.1 (specificity control) (⊘)], graded numbers of remaining viable cells were sensitized to a fixed number of BALB irradiated spleen cells over a 5-day period. The left panel illustrates the lytic activity at a 6:1 attacker/target ratio following sensitization of graded numbers of viable cells. The right panel shows the total lytic activity produced by these cells, a product of the specific lysis per 6×10^5 sensitized cells and the number of viable cells harvested from each group:

$$\text{Total lytic activity} = \frac{\text{lysis (6:1 ratio)} \times \text{cell yield/culture}}{5 \times 10^5}.$$

anti-Ly-1.2 pretreatment: (*a*) the increase was most apparent when high concentrations of T cells were sensitized and (*b*) it was substantially lower than would be predicted from the four- to fivefold enrichment of Ly-23$^+$ cells.

As noted previously (1), pretreatment with anti-Ly-2.2 or anti-Ly-3.2 abolished the ability of LN-T cells to generate a cytotoxic response. Thus, a cell population enriched twofold for Ly-1$^+$ T cells, and depleted of Ly-23$^+$ and Ly-123$^+$ T cells, is unable to generate appreciable lytic activity. (No impairment in the generation of killer activity resulted from pretreatment of control B6/Ly-2.1 congenic cells with anti-Ly-2.2, again confirming the specificity of this effect.)

II. The Contribution of Different Subclasses of Ly$^+$ Cells in LN to the Generation of Cytotoxic Activity. To estimate any possible contribution of Ly-1$^+$ and Ly-123$^+$ subclasses to the generation of killer activity by LNC, groups of 30×10^6 viable LNC were treated with different Ly antisera + C, washed 2 times and sensitized in vitro. In these experiments, the viable cell counts were not adjusted to equal the NMS controls, because we could thus better assess the contribution of each Ly subclass, present in the normal LN population, to the generation of killer activity mediated by Ly-23$^+$ effector cells (Fig. 2). Removal of Ly-23$^+$ and Ly-123$^+$ cells by treatment with anti-Ly-2 before sensitization (group C) abolished the ability of LN cells to generate a measureable lytic response. Removal of Ly-1$^+$ and Ly-123$^+$ subclasses by treatment of LNC with anti-Ly-1.2 before sensitization (group B) resulted in a slight decrease in the specific lytic activity generated, and a marked decrease in total lytic activity (the product of specific lytic activity $\times$ cell yield/well; see Materials and Methods). To determine whether the decrease in total lytic activity was due to depletion of Ly-1$^+$ cells or Ly-123$^+$ cells, we sensitized a mixture composed of cells treated with anti-Ly-1 and cells treated with anti-Ly-2 (group D). This mixed population was therefore depleted of Ly-123$^+$ cells. This mixture generated specific and total lytic activity approximately equal to that produced by NMS control cells (group A). Moreover, killer activity produced by this cell mixture was sensitive to anti-Ly-2.2 and resistant to anti-Ly-1.2 treatment performed after sensitization (Fig. 2, right-hand panel).

III. Demonstration that Ly-1$^+$ Cells do not Directly Contribute to the Cytotoxic Response. The above experiments indicate a synergistic interaction between Ly-1$^+$ cells and Ly-23$^+$ cells in the generation of Ly-23$^+$ killer cell activity. One explanation for these observations is that although Ly-1$^+$ cells do not contribute directly to the pool of cytotoxic effector cells, they amplify the generation of Ly-23$^+$ killer cells from Ly-23$^+$ prekillers. Alternatively, it is possible that some Ly-1$^+$ cells, in the presence of Ly-23$^+$ cells and alloantigen, differentiate to Ly-23$^+$ killer cells. This latter possibility was tested. B6 mice (Ly phenotype 1.2,2.2,3.2) were used as the source of Ly-1$^+$ cells (after treatment with anti-Ly-3.2). B6/Ly-2.1,3.1 congenic mice (Ly phenotype 1.2,2.1,3.1) were used as the source of Ly-23$^+$ cells (after treatment with anti-Ly-1.2). In this mixture, any Ly-1.2$^+$ cells that directly contributed to the generation of Ly-23$^+$ cytotoxic effector cells would be marked by expression of the Ly-3.2 antigen on the cytotoxic effector cell population. Table I shows that killer activity generated by this cell mixture was insensitive to anti-Ly-3.2. Since killer activity generated by untreated B6 cells *was* sensi-

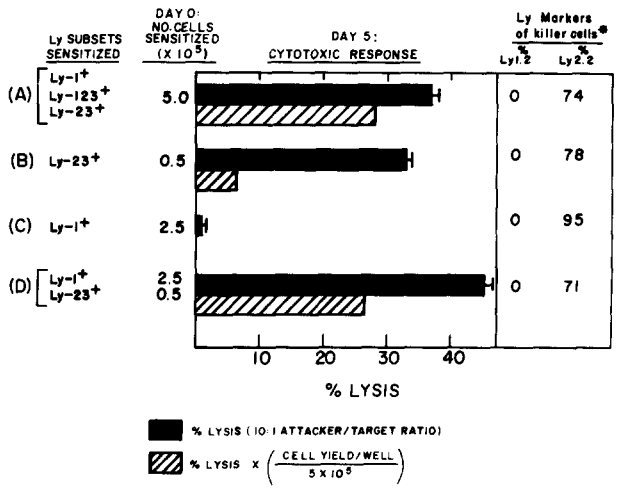

FIG. 2. The participation of different Ly subclasses in the generation of killer activity. Equal numbers of B6 lymph node cells were treated with NMS (group A), anti-Ly-1.2 (group B), or anti-Ly-2.2 (group C), in the presence of complement, and put into MLC *without* adjusting the viable cell counts to equal concentrations in all groups. Thus anti-Ly-1.2 removed Ly-1$^+$ and Ly-123$^+$ cells; anti-Ly-2.2 removed Ly-23$^+$ and Ly-123$^+$ cells. A mixture of these two selected cell populations was also put in MLC in the estimated normal ratio of 5:1, Ly-1$^+$:Ly-23$^+$ cells; this mixture is depleted of Ly-123$^+$ cells (group D). After 5 days in MLC, killer activity was measured (10, 5 and 2.5 attacker/target ratios). Lytic activity at these ratios exhibited linear (straight-line) dose response kinetics; the lytic activity at 10:1 attacker/target ratio is shown in the left-hand panel.

As shown in the right-hand panel (*), portions of all sensitized cell suspensions were treated after MLC with either NMS, anti-Ly-1, or anti-Ly-2 + C to determine the Ly phenotype of the killer effector cells generated; these data are expressed as percent reduction of killing activity (see Materials and Methods for details).

TABLE I

Evidence that the Amplifying Effect of Ly-1$^+$ T Cells is Not Due to Their Conversion to Ly-23$^+$ T Cells

Responder cells (treatment before MLC)	Cytotoxicity of effector cells, treated with:			
	Exp. 1		Exp. 2	
	NMS + C	Anti-3.2 + C	NMS + C	Anti-3.2 + C
(1) B6 (Ly-1.2,2.2,3.2) + NMS + C (control)	31	10	28	3
(2) B6 (Ly-1.2,2.2,3.2) + anti-3.2 + C → Ly-1$^+$ subclass (A)	6	3	1	0
(3) B6/Ly-2.1,3.1 + anti 1.2 + C Ly-23$^+$ subclass (B)	21	23	18	24
(4) 50% (A) + 50% (B)	28	30	39	44

Interpretation: Amplification of Ly-23$^+$ killing is seen by comparing groups 3 and 4 ([A + B] > B) although group 3 had twice as many Ly-23$^+$ cells. If the mechanism of this amplification were conversion of Ly-1$^+$ cells (A) to Ly-23$^+$ killers during the 5-day MLC, they would have acquired the Ly-3.2 phenotype (see group 2) and would have been susceptible to anti 3.2$^+$ C. They were not (see group 4).

tive to anti-Ly-3.2 (positive control), this experiment indicates that Ly-1$^+$ cells in the mixture do not differentiate to Ly-23$^+$ killer cells.

IV. The Effects of Sensitizing Different Subclasses of BALB Ly$^+$ Cells to B6 Alloantigens. The findings dealt with so far might be accounted for by supposing that clones of B6 lymphocytes possessing anti-*H-2^d* reactivity also express, for unknown reasons, Ly-23 surface determinants. In that case the general proposition could be made that different Ly phenotypes are associated with reactivities to particular antigens rather than with particular functions. We therefore tested the effects of Ly antisera on the response of BALB cells (Ly phenotype 1.2,2.2,3.2) to B6 cells (*H-2^d* anti-*H2^b*) (Fig. 3). Depletion of Ly-1$^+$

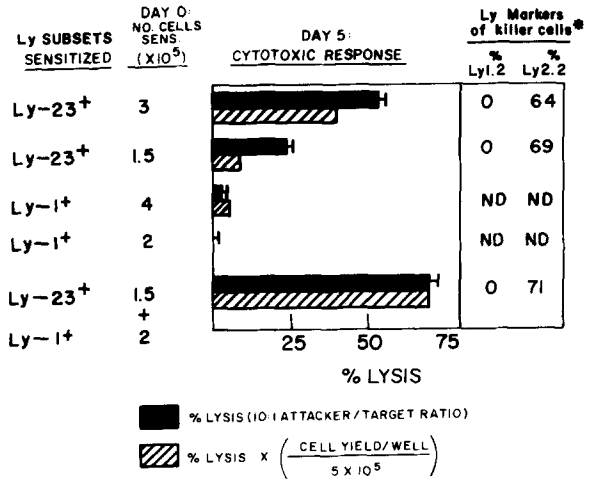

FIG. 3. The contribution of different Ly subclasses to the generation of *H-2^{d}αH-2^b* cytotoxic activity. BALB column-passed lymph node cells were pretreated with Ly antisera and sensitized with B6 (2,500 R) cells × 5 days in MLC. Pretreatment with anti-Ly-1.2 resulted in a T-cell population composed mainly of Ly-23$^+$ cells while treatment with anti-Ly-2.2 resulted in a cell population composed mainly of Ly-1$^+$ cells. Mixtures of these two cell populations produced lytic activity substantially greater than the sum of the activities produced by either cell population alone. The right-hand panel again shows the percent reduction in killer activity resulting from treatment of the sensitized cells after 5 days in MLC with Ly antisera + C (*).

cells resulted in a small decrease in generation of specific lytic activity and a substantial decrease in total lytic activity. Cells depleted of Ly-23$^+$ cells were unable to generate a significant cytotoxic response, as was the case after depletion of this subclass in the B6 anti-BALB response (Fig. 2). As with the previous *H-2^b* anti-*H-2^d* combination (Fig. 2), the additon of Ly-1$^+$ cells to Ly-23$^+$ cells markedly enhanced the generation of Ly-23$^+$ killer activity. While these tests tend to rule out the "antigen specificity" hypothesis, this deserves attention in further studies, because the antigens involved all belong to MHC systems.

V. The Requirement for Cooperative Interaction between Ly-1$^+$ T Cells and Ly-23$^+$ T Cells in the Generation of Maximal Cytotoxic Responses. Although the addition of Ly-1$^+$ cells to Ly-23$^+$ cells amplified the generation of Ly-23$^+$

killer activity, it was clear that Ly-23$^+$ cells could generate substantial killer activity in the absence of Ly-1$^+$ cells (Fig. 1). However, the dose response curve for these cells (Fig. 1) suggested that relatively small numbers of Ly-23$^+$ cells by themselves (comparable to their proportions in normal LN), generated considerably reduced activity. Therefore, we examined the effects of adding a fixed number of Ly-1$^+$ LN cells to different numbers of Ly-23$^+$ LN cells upon the generation of cytotoxic activity (Fig. 4). These experiments showed that the amplifying effects of Ly-1$^+$ cells were most evident when very small numbers of Ly-23$^+$ cells were used.

VI. The Effects of Removing Ia$^+$ Stimulator Cells upon Ly-1$^+$ Amplifier Activity in MLC. Since Ly-1$^+$ cells, in contrast to Ly-23$^+$ cells, have been shown to respond to *I* differences in MLC (1), we tested the effects of removing cells bearing Ia antigens from the stimulator cell population upon amplifier

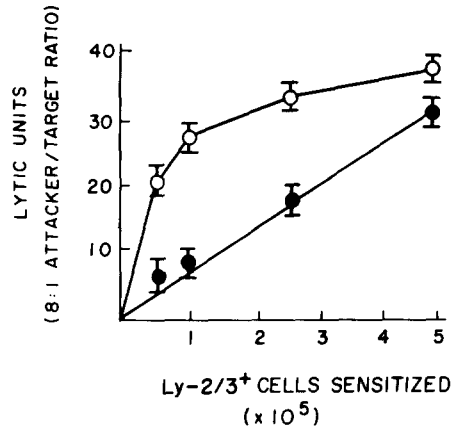

FIG. 4. The requirement for Ly-1$^+$ cells for the generation of optimal cytotoxic responses by small numbers of Ly-23$^+$ cells. 4 $\times$ 10^5 Ly-1$^+$ cells (obtained by treatment of B6 LN-T cells with anti-Ly-2.2) were added to different numbers of Ly-23$^+$ cells (obtained by treatment of LN-T cells with anti-Ly-1.2). Total lytic activity (see Materials and Methods) expressed as Lytic Units produced by different numbers of Ly-23$^+$ cells alone (●), compared with different numbers of Ly-23$^+$ cells mixed with 4 $\times$ 10^5 Ly-1$^+$ cells before in vitro sensitization (○), is shown.

activity of Ly-1$^+$ cells. To this end, Ly subclasses from BALB mice were stimulated by equal numbers of viable B6 spleen cells that had been treated with either anti-Beta or NMS (+ C). In Table II, the data for Group (II) show that the response of Ly-23$^+$ cells (population A) to B6 cells treated by anti-Beta was not increased by the addition of Ly-1$^+$ cells (population B), in contrast to the data for Group (I), in which NMS was substituted for anti-Beta, where Ly-1$^+$ cells (B) amplified substantially the lytic activity of the Ly-23$^+$ (A) population.

Discussion

These studies show that in B6 and BALB mice (Ly phenotype 1.2,2.2,3.2), peripheral lymphoid cells that have the capacity to generate killer activity to MHC alloantigens belong to a numerically small subclass of peripheral T

TABLE II

Evidence that Removal of Ia-Bearing Cells from the Stimulator Population in MLC Abolishes the Amplifier Effect of Ly-1$^+$ Cells

Antiserum (+ C) pretreatment of BALB responder cells:	Cell no. sensitized /well ($\times 10^5$)	Pretreatment of stimulator cells (B6/spleen cells–1,000 R)	Percent Lysis ± SE	Total lytic activity
Group I				
Anti-Ly-1.2	4	NMS + C	41 ± 1.5	61
Anti-Ly-1.2 (A)	2	"	22 ± 1.4	31
Anti-Ly-3.2 (B)	2	"	4 ± 0.8	4
(A) + (B)	2 + 2	"	56 ± 3.1	66
Group II				
Anti-Ly-1.2	4	Anti-β + C	23 ± 1.6	21
Anti-Ly-1.2 (A)	2	"	13 ± 1.0	12
Anti-Ly-3.2 (B)	2	"	3 ± 0.9	2
(A) + (B)	2 +2	"	10 ± 0.6	8

lymphocytes. This subclass, distinguished by the surface phenotype Ly-23$^+$, constitutes less than 10% of all peripheral T lymphocytes. But although these Ly-23$^+$ prekiller cells generate effector cells in the absence of other T-cell subclasses, the provision of Ly-1$^+$ T cells enhances the production of killer cell activity.

Since (*a*) Ly-1$^+$ cells do not develop killer activity alone, and (*b*) killer effector cells bear Ly-23 and lack Ly-1, it is likely that Ly-1$^+$/Ly-23$^+$ synergy can be ascribed to Ly-1$^+$ cells assisting Ly-23$^+$ cells to produce killer effector cells. An alternative explanation, that Ly-1$^+$ cells in the cell mixtures are somehow induced by Ly-23$^+$ cells to differentiate to Ly-23$^+$ killer cells, is rendered improbable by the experiments detailed in Table I.

The capacity of Ly-1$^+$ cells to amplify[2] an immune response is not limited to the generation of killer activity. Ly-1$^+$ cells also help B cells [defined more precisely as antibody-forming precursor cells (AFPC)] to produce antibody (1). On obvious difference in the interaction of Ly-1$^+$ cells with prekiller T cells as distinct from AFPC (B cells) is that in the former instance, sufficient numbers of prekillers can produce significant cytotoxic responses by themselves. In contrast, stimulation of large numbers of B lymphocytes with most antigens does not engender appreciable amounts of antibody. However, this difference in the requirement for T-cell help may not signify fundamental differences in the mechanisms of T-T and T-B interactions, but rather may stem from the more complex immunogenicity of allogeneic cells as compared with conventional protein antigens. This view is supported by the observation that a considerable

[2] For clarity, the enhancing effect of Ly-1$^+$ T cells upon the production of antibody by B cells will be referred to as "helper" function, according to accepted usage, while the enhancing effect of this subset upon the generation of Ly-23$^+$ killer cells will be referred to as "amplifier" function, although the mechanisms of both Ly-1$^+$ activities may be similar.

anti-*H-2* antibody response can be obtained in the absence of significant numbers of helper T cells (4).

We do not regard our experiments as definitive with respect to the specificity of the T-T interaction observed. However, the following two points are persuasive: (*a*) Ly-1$^+$ cells accounted for most of the proliferative response to an *I* region difference, whereas Ly-23$^+$ cells recognize *K* and *D* but not *I* region difference (1), and (*b*) Ly-1$^+$ amplifier activity was not seen when the stimulator cells were depleted of Ia(Beta)$^+$ cells. Both findings support the idea that Ly-1$^+$ amplifier cells respond to Ia determinants, while Ly-23$^+$ prekiller cells respond to *K* and *D* determinants. This is in accord with the observation that some *I* region differences between cells in MLC facilitate the development of cytotoxic responses against *H-2K* and *H-2D* differences (12, 13). An analogous finding has come from studies of the genetic basis of the cytotoxic response in human MLC (14).

The synergistic interaction between amplifier Ly-1$^+$ and prekiller Ly-23$^+$ cells described here may account for synergistic cell-mediated responses produced by mixtures of T cells from different lymphoid tissues, as well as for cell synergy between peripheral spleen T cells bearing different surface amounts of Thy-1.2 (5–11). These phenomena may reflect the combining of a cell population having a preponderance of the Ly-23$^+$ cells with populations having an excess of Ly-1$^+$ activity.

A summary of the biologic and immunologic properties of the three Ly subclasses of T lymphocytes defined thus far is given in Table III. Although the immunologic functions of Ly-1$^+$ and Ly-23$^+$ T cells are being defined, the role of Ly-123$^+$ cells is not established. In some cases, such as the response of BALB

TABLE III
Summary of Biologic Properties and Immunologic Activities of Subclasses of Ly$^+$ T Lymphocytes

Biologic features	Test criterion	Ly subclass		
		Ly-123$^+$	Ly-1$^+$	Ly-23$^+$
Ontogeny	Time of appearance after birth (1)	Early	Late	Late
Short-term dependence on thymus in adult life	Representation of subclass in spleen 4 wk postthymectomy (1)	Dependent	Independent	Independent
Immunologic functions				
Alloantigen recognition in MLC	Thymidine incorporation vs. MHC incompatibility (1)	+	+	+
	MLC vs. *I* region incompatibility (1)	–	+	–
Killer potential	Generation of killer cells in vivo (1)	(–)*	–	+
	Generation of killer cells in vitro (1)	(–)*	–	+
Killer activity	After generation in vivo (1,2,15,16)	–	–	+
	After generation in vitro (1)	–	–	+
Helper-amplifier potential				
Interaction with antibody-forming precursors (B cells)	Adoptive early primary antibody response to SRBC (1)	(–)*	+	–
Interaction with killer precursors (Ly-23$^+$ T cells)	Sensitization in vitro (Figs. 2, 3)	–	+	–

* Although Ly-123$^+$ cells in LN do not appear to make a positive contribution to the early phase of these responses, the role of these cells in the later phases of these responses has not been investigated.

spleen cells to B6 antigen, removal of Ly-123$^+$ cells causes significant reduction in the generation of killer activity (data not shown). In other responses, such as that of B6 LN cells to BALB antigen, Ly-123$^+$ cells appear to make no positive contribution to the killer response (Fig. 2). The rapid decline of this subclass after thymectomy suggests that Ly-123$^+$ cells are relatively short-lived. This cell population may exert immunoregulatory effects, or may represent a transitional population that can give rise to mature Ly-1$^+$ and Ly-23$^+$ progeny; these possibilities are not mutally exclusive. We are studying whether such a regulatory function is demonstrable, and if so, whether it is influenced by genes in the *I* region.

These results and those of previous studies of the Ly phenotypes of T cells (1, 2) summarized in Table III, indicate that peripheral T lymphocytes in unimmunized mice comprise subclasses of cells that have been programmed during differentiation to express different immunologic functions.

Summary

Lymphocytes from BALB/c or C57B6/6 mice that develop killer activity to alloantigens belong to the numerically small Ly-23 subclass of peripheral T cells, distinguished by selective expression of Ly-23 determinants on their surfaces. The maturation of these cells to killer cells can be amplified by Ly-1$^+$ cells, which do not themselves contribute to the killer cell pool. This amplification was abolished by excluding Ia("Beta")$^+$ cells from the stimulator population during mixed lymphocyte culture (MLC), suggesting that amplification is due to selective recognition of *I* region antigens by Ly-1$^+$ cells, a conclusion already drawn from our previous evidence that Ia differences activate Ly-1$^+$ cells but not Ly-23$^+$ cells. These and other experiments indicate that amplification of killer cell production in vitro by Ly-1$^+$ cells is not due to their conversion to Ly-23$^+$ cells during MLC, but to their ability to recognize major histocompatibility complex determinants not recognized by Ly-23$^+$ cells.

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