Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5667–5671, December 1977
Immunology

Ly phenotype of T cells cytotoxic for syngeneic mouse mammary tumors: Evidence for T cell interactions

(thymus dependency/tumor immunology/*in vitro* assay)

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Communicated by Lewis Thomas, September 12, 1977

ABSTRACT **Specific cell-mediated cytotoxicity (CMC) of lymph node cells from immunized C3Hf mice, against syngeneic C3H/Umc mammary tumor cells, assayed *in vitro*, is effected by T lymphocytes. This CMC response is biphasic, with an early peak attained within 6 hr and a second major peak beginning at about 18 hr. Effector cells of both the early minor and late major phases of the response belong to the Ly23 set. Other T cell sets evidently play no part in the early effector response. But specifically activated Ly1 cells help or amplify the major late-phase response. Nevertheless, the mixture of specifically activated Ly1 and Ly23 sets still does not completely reconstitute the late response, which implies that the Ly123 set is also needed for maximal expression of CMC in this system. These Ly123 cells must come from specifically immunized donors. It appears, therefore, that maximal CMC is achieved by the participation of specific Ly123 cells which in the late phase directly or indirectly give rise to Ly23 killer cells. Thus, although killing of syngeneic mammary tumor cells in the CMC assay is invariably effected by cells of the Ly23 set, specifically activated cells of the Ly1 set, and probably of the Ly123 set also, are participants in the interactions needed to produce a maximal CMC response.**

Thymus-derived (T) lymphocytes mediate several immune functions, including cytotoxicity against specific target cells *in vitro* (1). T cells with different functions have distinct surface markers of the Ly series (1–6). The surface phenotype of the T cell set that destroys allogeneic target cells is $Ly1^-$:$Ly23^+$ (Ly23 cells) (3–6). However, the phenotype of T cells capable of destroying syngeneic tumor targets, presumably by reacting against tumor-associated transplantation antigens, has been reported as $Ly1^+$:$Ly23^+$ (Ly123 cells) (7).

One factor that must be considered in comparing cell-mediated cytotoxicity (CMC) for allogeneic and tumor-associated systems is the difference in the length of time involved in the respective assays. The allogeneic assays are short-term (3–4 hr) and display single-hit kinetics, and the frequency of reactive cells is relatively high (8). In most syngeneic systems, long-term assays (20 hr or more) are required, the kinetics of which have not been defined (9, 10). Our present analysis of long-term assays for syngeneic tumor cytotoxicity shows that they involve participation by at least two sets of T cells. The $Ly1^+$:$Ly23^-$ (Ly1) population, which is not itself cytotoxic, augments the generation of Ly23 cytotoxic T cells. Thus, although CMC against syngeneic mammary tumor cells is mediated by Ly23 cells at all stages of the assay, as we shall show, Ly1 cells, and probably Ly123 cells, are needed to implement a maximal Ly23-mediated effector response.

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MATERIALS AND METHODS

Animals and Tumors. The C3Hf and C3H mice used are derived from the colony of the late Dr. J. J. Bittner at the University of Minnesota (Umc sublines), and have been bred by one of us (O.S.) since 1973 (10). Mice used for the production of Ly antisera are described in ref. 11. All the present studies were done with MT.1, a spontaneous C3H/Umc mammary adenocarcinoma described in ref. 10. The PO.4 tumor referred to in Table 3 is a polyoma-induced mammary tumor of C3Hf origin, which is not crossreactive with MT.1 (also described in ref. 10).

Immunizations. Two- to four-month old C3Hf females were immunized by injection of 5×10^5 viable tumor cells in one or both hind footpads, followed by excision of the growing tumor 15–20 days later. Cells from the regional inguinal and popliteal lymph nodes were obtained from these animals 7–9 days after excision, which is the optimal timing for the maximal T-dependent cytotoxic response (10).

Target Cells. An established cell line derived from MT.1 (10) was used in all the *in vitro* assays. The MT.1 cells were passed serially *in vitro* in T-60 plastic flasks (Falcon Plastics, Los Angeles, CA) with culture medium made of RPMI 1640 (Grand Island Biological, Grand Island, NY) supplemented with 15% fetal bovine serum and insulin at 10 μg/ml.

Cell-Mediated Cytotoxicity Assay. The procedure is similar to that described in refs. 9 and 10. Monolayer cultures of target cells in T-60 flasks were incubated for 20–24 hr with 250 μCi of [^{3}H]proline (L-[2,3-^{3}H(N)]proline, with specific activity of 40–50 Ci/mmol, from New England Nuclear Corp., Boston, MA) in 5 ml of complete Hanks' minimal essential medium (without nonessential amino acids, i.e., without proline) with 15% fetal bovine serum. The cultures were then washed three times at 37° with complete minimal essential medium containing 15% fetal bovine serum and 2% nonessential amino acids. The cells were detached with 0.05% Tryptar (Armour Pharmaceutical, Chicago, IL) in Puck's saline A (0.02% EDTA used on occasions) and the cell concentrations were adjusted so that 10 μl would contain 1000 cells. Ten microliters of cell suspension was distributed with a microliter syringe (Hamilton Co., Whittier, CA) into the wells of Falcon microtest plates II (no. 3040, Falcon Plastics, Los Angeles, CA) prefilled with 0.1 ml of culture medium. Effector cells were added 6 hr later in 0.1 ml of culture medium at 100:1 lymphocyte/target ratios. The plates were then incubated at 37° in humidified 5% CO_2 in air for 30 hr, unless otherwise indicated. At the end of the

Abbreviations: α, anti-; C, complement; CMC, cell-mediated cytotoxicity; E:T ratio, effector-to-target-cell ratio; Ly1, $Ly1^+$:$Ly23^-$; Ly23, $Ly1^-$:$Ly23^+$; Ly123, $Ly1^+$:$Ly23^+$; T, thymus-dependent (lymphocytes).

Table 1. Effect of pretreatment of effector lymphocytes with anti-Ly or Thy-1 sera and C on syngeneic target cell destruction

Cells*	Pre-treatment†	cpm ± SEM in target cells‡	% CMC	% reduction of CMC
None	—	4832 ± 433	—	—
Normal	Diluent only	4360 ± 306	10	—
Immune	Diluent only	1321 ± 187	70	—
Immune	C control	1369 ± 209	69	2
Immune	α Ly-1.1	3227 ± 419	26	63
Immune	α Ly-2.1	3814 ± 459	13	82
Immune	α Thy-1.2	3837 ± 340	12	83

* Popliteal lymph node cells from normal or immune C3Hf 40-day-old females. For details on immunization against C3H MT.1, see *Materials and Methods.*

† Lymph node cells incubated for 45 min with 1:10 dilutions of sera and rabbit C. For details on cytotoxicity and antisera, see *Materials and Methods.* The percent of lysed cells was 50, 20, and 75, respectively, for αLy-1.1; αLy-2.1, and αThy-1.2.

‡ cpm remaining in target cells after 30 hr of incubation, 500 MT.1 target cells prelabeled with [^{3}H]proline and effector cells at 100:1 ratio.

incubation period, the plates were inverted and slightly shaken to remove medium and effector cells. The plates were then submerged three times in phosphate-buffered saline with 5% fetal bovine serum at 37° (a large volume being used and changed after every wash), emptied, and left to dry. When dry, the plates were sprayed lightly with Fluoro-glide (Chemplast, Inc., Wayne, NJ) and the bottoms of the wells were punched out by the use of a hand-operated cam punch press (Rope Whitney, Rockford, IL) with a cutting die 6.528 mm in diameter and a punch 6.350 mm in diameter. The punched bottoms were transferred to scintillation vials and 0.3 ml of Hyamine hydroxide (Packard Instrument Co., Downers Grove, IL) was added to each flask. After 30 min, 10 ml of toluene with scintillation fluid (Packard Permablend I, Packard Instrument Co.) was added. The vials were kept at 4° for at least 24 hr before measuring radioactivity (to ensure complete solubilization of target cell protein). All the tests were performed in three to five replicates. The results are expressed as mean cpm ± SEM or as percent cytotoxicity calculated according to the formula 100(1 − A/B), in which A = target cell cpm remaining after incubation with immune effector cells, and B = target cell cpm remaining after incubation with nonimmune effector cells.

Serological Reagents and Procedures. The Ly reagents used, and relevant serological procedures, are described in ref. 11 [for description of the anti-Thy-1.2 (αThy-1.2) serum see also refs. 9 and 10]. These standard procedures were used in the experiments described in Tables 1 and 2 and also to select the Ly1 or Ly23 populations referred to in Tables 3 and 4. In Fig. 2, the procedure was different, because the complement (C)-dependent cytotoxicity technique was performed directly on the microwells where CMC was taking place. In selected wells of the Falcon II microtest plates, at different times (0–24 hr) after initiation of the 30-hr CMC assay, most of the culture fluid was removed with a pasteur pipette and replaced with 0.025 ml of C and 0.025 ml of αLy sera at a predetermined dilution. The trays were then placed for 45 min at 37° for the C-dependent cytotoxicity to be completed, after which the wells were filled with 0.35 ml of culture media, and incubation was continued to complete the standard total of 30 hr. The 0.35 ml of culture medium produces an additional 20-fold dilution of antiserum + C during the remainder of the assay period. Control wells were: (*i*) treated in the same manner but without reagents (i.e., only with culture media), and (*ii*) with C alone. The Ly antisera were titrated by their addition to the CMC assay, at dilutions of 1:20 to 1:1000, at intervals of 6, 18, and 24 hr after initiation, and measuring CMC at 30 hr, as well as by the conventional cytotoxicity assay based on exclusion of trypan blue (11). The useful endpoint titer for our pool of αLy-1.1 was 1:1000, and 1:200 for the αLy-2.1; these were the dilutions selected for use in experiments.

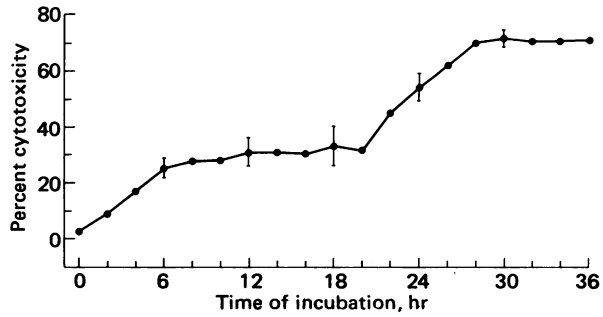


FIG. 1. Time course of cell-mediated cytotoxicity (CMC) of immune C3Hf lymph node cells against syngeneic C3H MT.1 mammary adenocarcinoma target cells. Target cells were prelabeled with [^{3}H]proline and 1000 cells were plated per well. The effector:target cell (E:T ratio) used for every point was 100:1. Each point represents the values of triplicate plates (four wells per point per plate, i.e., a total of 12 values per point) in a single experiment. Vertical bars indicate standard error of the mean percent cytotoxicity. Comparable biphasic curves were observed in six additional experiments.

RESULTS

Effect of Pretreatment of Cytotoxic T Cells with αLy-1 and αLy-2 Antisera. Table 1 shows an example of the results obtained when immune lymph node cells are pretreated *in vitro* with αLy-1, αLy-2, or αThy-1 antisera and C (which lysed 50–55, 20–25, and 60–70% of the cells, respectively) on CMC against syngeneic C3H mammary adenocarcinoma cells *in vitro* (after 30 hr incubation at 100:1 effector-to-target, E:T, ratio). As shown in previous work (10), the response is mediated by T cells as indicated by the marked effect of the αThy-1 treatment. CMC was equally affected by pretreatment of the effector T cells with αLy-1 or αLy-2 (63 and 82% reduction of CMC, respectively). These results seem to support the report by Shiku *et al.* (7) that the effector cells in syngeneic tumor systems are Ly123.

Time Course of the CMC Response. Before trying to determine the Ly phenotype of the effector T cells in our system, it seemed imperative to determine the kinetics of the response. Fig. 1 shows the percent CMC when measured at 2-hr intervals from 0 time to 36 hr of incubation (at 100:1 E:T ratios). The response is biphasic, with an early peak at 6 hr, which levels off for approximately 12 hr and is followed by a second peak that reaches its maximum after 24–30 hr of incubation, with no further increment. This biphasic curve was observed in every instance in seven different experiments, of which Fig. 1 is a representative example.

The Late CMC Peak Is Mediated by Ly23 T Cells. Fig. 2 shows the effects of selective T cell elimination at different times during the assay (0, 6, 12, 18, and 24 hr), the response then being allowed to continue for the usual total of 30 hr. The most notable effect was that although both αLy-1 and αLy-2 sera lowered the response when applied during the early phase, most of the effector cells active during the 18- to 24-hr period were insensitive to αLy-1 serum, and therefore presumably were of the Ly23 phenotype. Three additional experiments gave similar

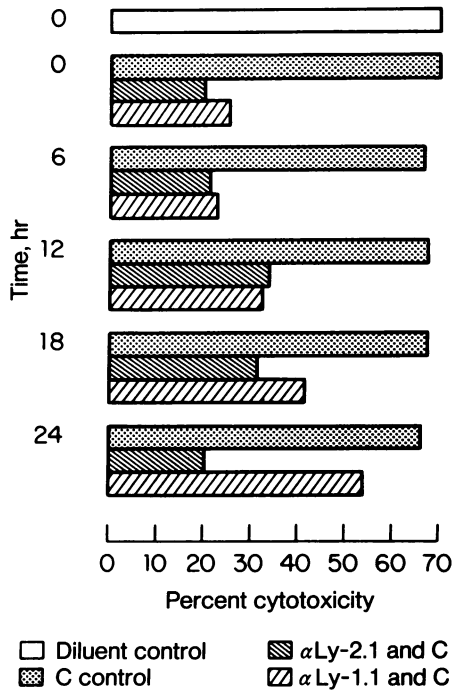


FIG. 2. Effect of treatment of effector cells with α-Ly sera and complement (C) on CMC of C3Hf immune lymph node cells against syngeneic C3H MT.1 mammary adenocarcinoma target cells. The E:T ratio used was 100:1 and the total incubation time was 30 hr. *Time* indicates the time at which the treatment of the effector cells with α-Ly and C was performed directly on the plates (see *Materials and Methods*) (0 indicates 10 min after beginning of the CMC assay; 6, 12, and 24 hr indicate the times within the cytotoxic assay of 30 hr at which the α-Ly and C was added).

results. To test further the specificity of the reduction produced by αLy-2 serum during the later phase, two portions of αLy-2.1 serum were absorbed respectively with: (*i*) T cells from C57BL/6/Ly-2.1 mice, and (*ii*) T cells from C57BL/6/Ly-2.2 mice. The former absorbed antiserum failed to abolish CMC activity, whereas the latter absorbed antiserum abolished CMC activity, as before. Thus, the CMC reduction produced by αLy-2.1 serum in the late phase is due specifically to the elimination of Ly23 cells.

The Early CMC Peak Is Also Mediated by Ly23 T Cells. Because the early events of the response are not adequately defined by treating with αLy serum at different time points and allowing the test to continue for its full 30-hr duration (see Fig. 2), we tested the effects of selecting with the αLy sera and measuring CMC at 6 and 12 hr after incubation instead of 30 hr. Table 2 shows that the early peak of CMC, observed at 6 and 12 hr, was also exclusively mediated by Ly23 cells; elimination with αLy-1 leaves the small early CMC peak intact while αLy-2 abolished it. Thus, both the early peak (Table 2), and the late CMC peak (Fig. 2), are mediated by Ly23 cells.

Although Ly3 phenotypes were not ascertained in the experiments described above, we use the notation Ly123, Ly1, and Ly23 in relation to the three known T cell Ly phenotypes (1, 4) because no discrepancies in expression of Ly2 and Ly3 have so far been reported (as far as is known, T cells express both or neither). To verify this in the present system, we ran tests with αLy-3 serum. Table 2 shows good concordance between the effects of αLy-2.1 and αLy-3.2 eliminations, indicating that the effector cell is Ly23.

Ly1 Cells Act as Helpers. Although both the early (6 hr) and the late (24 hr) peaks of CMC are mediated by Ly23 T cells, it

Table 2. Effect of pretreatment of immune C3Hf lymphoid cells with anti-Ly reagents and C on cytotoxicity against C3H MT.1 target cells

Treatment of cells*	% cytotoxicity at different end points (in hr)†		
	6	12	30
Diluent	27	32	69
C control	28	31	70
αLy-1.1	28	28	20
αLy-2.1	3	4	18
αLy-3.2	3	3	16

* Lymph node cells from C3Hf mice immunized against MT.1 (C3H), obtained 9 days after immunization. E:T ratios were 100:1. All treatments were done at 0 time.

† Target cells alone: 5933 ± 340 cpm; target cells plus normal C3Hf cells: 5427 ± 399 cpm.

is apparent that early removal of the Ly1$^+$ cells (Ly1 and Ly123) reduces the final CMC, especially the second peak (Fig. 2). Initial elimination with either Ly antiserum reduces overall CMC measured 30 hr later (Table 3). The Ly1 cells alone are not cytotoxic (third line of Table 3), whereas the Ly23 cells (fourth line of Table 3) have a low cytotoxicity, supporting the view that the early CMC peak is mediated by the Ly23 set (Table 2). Mixture of the selectively depleted populations (Ly1 set + Ly23 set; lacking the Ly123 set) partially restores the CMC response measured 30 hr later (44% CMC, fifth line Table 3, compared with 70% by the unfractionated cells, second line Table 3). Table 3 (last four lines) also shows that both the Ly1 and the Ly23 populations must come from donors specifically immune against the mammary tumor target; Ly1 and Ly23 sets from normal C3Hf mice, or from C3Hf mice immunized against a noncrossreacting C3Hf tumor, do not reconstitute (respectively) the CMC response of either specific immune population alone.

The reconstitution experiments in Table 3 imply that both Ly1 and Ly23 sets are required for optimal CMC. However, the comparisons in such experiments were favoring the mixtures

Table 3. Reconstitution of CMC by mixing effector cells pretreated with anti-Ly-1.1 or anti-Ly-2.1 and C

Cell types*	cpm†	% CMC	% reduction
Unfractioned (diluent)	1438	73	—
Unfractioned (C control)	1599	70	5
Ly1 cells (αLy-2.1 + C)	4923	6	91
Ly23 cells (αLy-1.1 + C)	4030	23	68
Ly1 + Ly23	2934	44	37
Ly1 + Ly23 (normal)	4830	8	89
Ly1 (normal) + Ly23	4001	24	66
Ly1 + Ly23 (Imm. PO.4)	4874	7	90
Ly1 (Imm. PO.4) + Ly23	3989	24	66

* All cells were derived from C3Hf mice immunized against MT.1 (C3H). In parentheses is the pretreatment. Normal cells were derived from nonimmune age- and sex-matched C3Hf controls; Imm. PO.4 indicates that the cells were derived from C3Hf animals immunized with a polyoma-induced mammary tumor of C3Hf origin (PO.4; see ref. 10 for details on this tumor). The E:T ratios used were 100:1 for the first four groups (unfractioned, Ly1, or Ly23 immune cells), and 200:1 (i.e., 100:1 of each type) for the different Ly1 and Ly23 mixtures. The controls of target cells with normal C3Hf lymph node cells were prepared at 200:1 ratios.

† Target cells alone were 5337 ± 400 cpm; target cells plus normal C3Hf lymph node cells at E:T ratios of 200:1 were 5201 ± 333 cpm. All assays were incubated for 30 hr.

Table 4. Reconstitution of CMC by mixing purified Lyl and Ly23 immune cells

Cell types*	E:T ratios	% CMC†
Unfractioned (C control)	200:1	79
	150:1	75
	100:1	70
	50:1	56
Lyl cells	200:1	6
	100:1	6
	50:1	4
Ly23 cells	200:1	48
	100:1	24
	50:1	19
Lyl + Ly23 cells	(100 + 100):1	48
	(50 + 100):1	46
	(100 + 50):1	50
	(50 + 50):1	42

* All cells were derived from regional lymph nodes of C3Hf mice immunized against MT.1. See *Materials and Methods* and Table 1 for details on preparation of the purified Lyl and Ly23 cells.

† All assays were incubated for 30 hr. Target cells alone were 5987 ± 350 cpm; target cells plus normal C3Hf lymph node cells at 200:1 ratios were 5777 ± 199 cpm; target cells plus normal C3Hf lymph node cells at 100:1 ratios were 5703 ± 247 cpm. These values indicate cpm ± SEM in remaining target cells prelabeled with [^{3}H]proline.

(prepared by mixing the two cell types at 100:1 E:T ratios of each class, i.e., a total of 200:1 E:T ratios). A more detailed study of mixtures at different ratios is presented in Table 4. When tested individually at ratios ranging from 50:1 to 200:1, it was apparent that the Lyl cells were not cytotoxic even at the highest ratio, while the Ly23 cells were cytotoxic only, although less so than the unfractionated population. The mixtures of Lyl and Ly23 cells gave a substantial degree of restoration of CMC, higher than the sum of the two cell types alone (especially at the lower E:T ratios; last three lines of Table 4), but this did not in any instance reach the level of the unfractionated cells. Table 4 also shows that even at the 50:1 ratio the proportion of Lyl and Ly23 cells is still optimal, because the values are comparable to those obtained with 100:1 ratios. The addition of untreated normal lymph node or spleen cells to the mixtures of immune Lyl and Ly23 sets (at 50 or 100:1 ratios) as a source of Ly123 cells did not change the percent CMC of the mixture (CMC ranged from 43 to 50%; data not shown in Table 4).

DISCUSSION

This study shows that long-term assays designed to measure CMC against syngeneic tumors *in vitro* are complex (Fig. 1) because they involve not only the effector response but also terminal steps in T cell differentiation. It is now clear that the generation of maximal CMC against syngeneic adherent tumor targets, in an assay extending over a period as long as 30 hr, requires interactions among different T cell sets. Therefore, earlier experiments, showing that CMC could be abolished by pre-elimination with either Lyl or Ly2 antiserum, should not be taken to indicate that the cytotoxic effector cell belongs to the Ly123 set. The issue was clarified in our experiments by eliminating different Ly sets at different intervals during the 30-hr incubation period, and alternatively by terminating the assay within 6 or 12 hr of Ly selection. It is then seen that cytotoxic effector cells at all phases of this syngeneic CMC response against mammary tumor cells belong to the Ly23 set, as also is the case with allogeneic target cells and autogenous hapten-modified target cells.

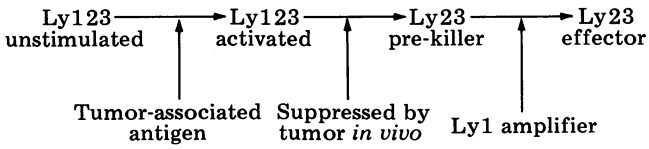
The Lyl set is not itself cytotoxic, but its addition to the Ly23 set substantially reconstitutes the CMC response (Tables 3 and 4), indicating a helper or amplifier interaction with Ly23 pre-killer cells. Both sets must come from immunized donors and therefore the Lyl helper may induce "non-lytic secondary T cells" (12) or cause specific cytotoxic memory T cells to become cytotoxic effector Ly23 cells (8, 12). Our unpublished data also show that cooperation is seen when Lyl helper cells are added to Ly23 cells as late as 18 hr after initiation of the assay, suggesting rapid induction that may not entail proliferation of the Ly23 cells but rather may entail a final differentiative step. The cytotoxic activity of mixtures of different ratios of Lyl and Ly23 cells indicates that even at the lowest ratios (Table 4) there is true synergy, the response being always greater than the additive effects of the two populations used in the mixture. However, the use of large numbers of the Ly23 population (200:1 E:T ratio) may circumvent the need for Lyl help (Table 4) because the level of cytotoxicity reached by the Ly23 cells alone is comparable to that of the mixtures of lower ratios of Ly23 cells and Lyl helper cells.

The fact that reconstitution of CMC does not attain the full response of the unselected population signifies a role also for the Ly123 set that is lacking from mixtures of Lyl and Ly23 sets. The augmenting role of Ly123 cells can be explained as a contribution by pre-killer cells in the Ly123 pool, which has been shown to give rise to cytotoxic Ly23 cells against hapten-modified syngeneic target cells (1). Because mixtures of immune Lyl and Ly23 plus normal untreated spleen or lymph node cells (which contain Ly123 pre-killer cells) did not produce an increased cytotoxic response, the source of Ly123 cells must be an immunized donor. Thus, *de novo* sensitization of this set is not apparent within the 30-hr period of the assay.

To recapitulate: Full CMC expression in this system requires (*i*) a specific interaction between Lyl and Ly23 sets that augments the generation of Ly23 killers from the nonlytic secondary or memory T cells, and (*ii*) probably the differentiation of specific Ly123 cells to Ly23 killers, triggered by antigen or by specific Lyl cells.

Interactions of T cells in generating an allogeneic CMC response have been shown by mixing thymocytes and peripheral T cells (13–15) and by mixing T cells from different peripheral sources (15). For allogeneic targets the precursor-effector and effector T cells generated *in vitro* have the phenotype Ly23, and the amplifier set Lyl (1, 4). For hapten-modified autogenous targets, the effector cell is again Ly23, but pre-killers of Ly123 type are demonstrable (1). This need not imply an essential difference between the two systems; it may simply mean that full expression of CMC in weaker systems necessitates recruitment of more primed cells from an earlier compartment in the sequence of differentiative steps that supplies the cytotoxic Ly23 effector cell.

Finally, a provocative hypothesis in connection with the data reported here is that in certain tumor systems the transition of Ly123 cells into effector Ly23 cytotoxic cells may be inhibited *in vivo*, with a consequent preponderance of noneffector Ly123 precursors, according to a model such as this:



We wish to thank Michael Cuttito and Robert Bohnenberger for their technical assistance and Linda Stevenson for her help in preparing this manuscript. This work was supported by Grants CA-08748, CA-16889, CA-17818, and RR-01001 and Contract CB-64031, awarded by the National Institutes of Health, Department of Health, Education and Welfare.

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