Brief Definitive Report

Ly PHENOTYPE OF CYTOTOXIC T CELLS FOR SYNGENEIC TUMOR*

By HIROSHI SHIKU, TOSHITADA TAKAHASHI, MICHAEL A. BEAN,‡ LLOYD J. OLD, AND HERBERT F. OETTGEN

(From the Memorial Sloan-Kettering Cancer Center, New York 10021)

Functionally distinct subsets of thymus-derived (T) cells differ with regard to their Ly surface phenotype (1–3). Peritoneal T cells of C57BL/6 (B6) mice which are cytotoxic for BALB/c target cells are Ly-1$^-$, Ly-2/3$^+$, whereas B6 spleen cells which perform a "helper function" in the production of antibody to sheep red blood cells are Ly-1$^+$, Ly-2/3$^-$. We report here that cytotoxic T cells for syngeneic tumor cells belong to a different T-cell subset than cytotoxic cells for allogeneic target cells.

Materials and Methods

Immunization and Microcytotoxicity Assay. B6 sarcoma Meth 4, induced by subcutaneous injection of methylcholanthrene, was in its 5th to 15th transplant generation in syngeneic mice during these studies. B6 female donors received four weekly intraperitoneal injections of 5×10^6 irradiated (10,000 rads) Meth 4 cells or 5×10^6 B6 or BALB/c spleen cells. Peritoneal cells were harvested 3 days after the last immunization and were incubated with [^{3}H]proline-labeled monolayer target cells for 24 or 36 h. (In our experience, lymphocyte cytotoxicity in syngeneic tumor systems requires a longer incubation period for maximal target cell destruction than in allogeneic reactions [12–20 h incubation].) Detached target cells and effector cells were removed by washing and the radioactivity (counts per minute) of the remaining cells was measured. (For details of microcytotoxicity assay, see reference 4.)

Pretreatment of Cytotoxic Peritoneal Lymphocytes with Antiserum and Complement (1, 2). Peritoneal cells from groups of five B6 mice immunized with either Meth 4 cells (immune) or syngeneic spleen cells (nonimmune) were pooled. Peritoneal lymphocytes were obtained by removing glass-adherent cells, as described previously (1, 4). For pretreatment with antiserum and complement (C), equal volumes of (*a*) lymphocytes (5×10^6/ml), (*b*) appropriately diluted antiserum (titers were determined by standard C-dependent cytotoxicity assays and the dilutions chosen for tests covered the range of maximal cytotoxicity for peritoneal lymphocytes), and (*c*) C (selected rabbit serum [1/15] for mouse antisera, or guinea pig serum [1/3] for rabbit antisera) was mixed and incubated for 45 min at 37°C. The medium was Eagle's balanced salt solution with 2.5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For control purposes, antisera against reciprocal Ly or Thy-1 alleles, or normal rabbit serum (in the case of rabbit antisera) were used. Specificity was further confirmed by absorption tests with thymocytes from mice of congenic Ly strains (5). After pretreatment with antiserum and C, washed lymphocytes (2×10^5 or 4×10^5 viable lymphocytes per well) were incubated with [^{3}H]proline-labeled target cells (1×10^3 per well) for 36 h at 37°C in 5% CO_2 in air. "Percent cell-mediated cytotoxicity (CMC)" was calculated according to the formula $[1 - (a)/(b)] \times 100$, where (*a*) and (*b*) represent counts per minute of remaining target cells after incubation with immune lymphocytes (*a*) or nonimmune

* Supported by Grant CRI-287 of the Cancer Research Institute, Inc. and grants CA-08748 and CA-17404 of the National Cancer Institute.

‡ Present address: Virginia Mason Research Center, Seattle, Wash.

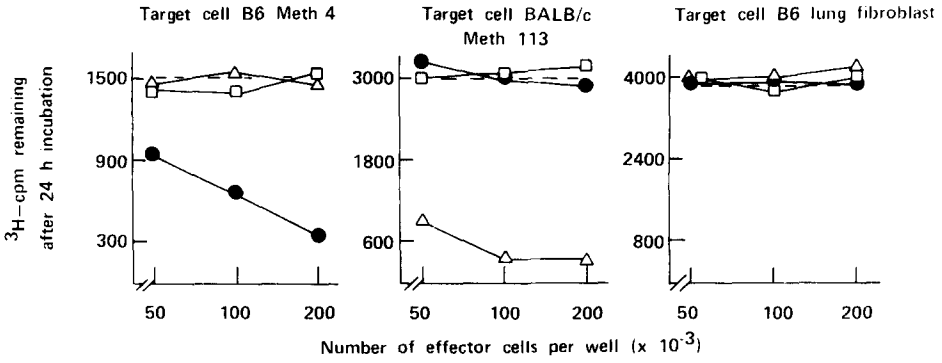

FIG. 1. B6 donors received 4 weekly intraperitoneal injections of 5×10^6 (*a*) B6 sarcoma Meth 4 cells (10,000 rads), (*b*) B6 spleen cells, or (*c*) BALB/c spleen cells. Peritoneal cells harvested 3 days after the last injections were incubated with [^{3}H]proline-labeled monolayer target cells. (---), no peritoneal cells; (●—●), peritoneal cells from B6 mice injected with irradiated B6 sarcoma Meth 4; (△—△), peritoneal cells from B6 mice injected with BALB/c spleen cells; and (□—□), peritoneal cells from B6 mice injected with B6 spleen cells.

lymphocytes (*b*). Percent reduction of CMC resulting from pretreatment of peritoneal lymphocytes was calculated as $[1 - (A)/(B)] \times 100$, where (A) is percent CMC (see above) of lymphocytes pretreated with test antisera and C, and (B) is percent CMC of lymphocytes pretreated with control serum and C.

Results and Discussion

Fig. 1 shows cytotoxic assays with peritoneal cells from B6 mice immunized with the syngeneic methylcholanthrene-induced sarcoma, Meth 4. These cells destroyed Meth 4 cells, but not B6 lung fibroblasts or BALB/c sarcoma Meth 113 cells (Fig. 1). Peritoneal cells from B6 mice immunized with BALB/c spleen cells were cytotoxic for BALB/c target cells, but not for Meth 4 cells. The definition of surface antigens of chemically induced tumors is not sufficiently advanced to say which antigens are involved in this syngeneic cell-mediated killing. Current evidence points to surface antigens related to murine leukemia virus (MuLV), rather than the individually distinct antigens demonstrable by transplantation techniques, since Meth 4 is MuLV$^+$ and peritoneal lymphocytes from B6 mice immunized with Meth 4 cells lyse three other MuLV$^+$ B6 sarcomas.

To analyze the surface phenotype of syngeneic cytotoxic cells, subpopulations of peritoneal lymphocytes were prepared by eliminating cells marked by one or another T- or B-cell surface antigen through the use of cytotoxic antiserum and C (1, 2, 5). Pretreatment with antisera against Thy-1, mouse-specific T-lymphocyte antigen (MSLA) or Ig showed that the cytotoxic cells for syngeneic Meth 4 cells, as in our earlier experiments with allogeneic target cells (1, 4, 6), have a Thy-1$^+$, MSLA$^+$, Ig$^-$ phenotype (Table I). Pretreatment of immune B6 lymphocytes (phenotype Ly-1.2,2.2,3.2) with anti-Ly-1.2, anti-Ly-2.2, or anti-Ly-3.2 sera reduced their cytotoxicity for Meth 4 cells (Table II). Additional confirmation of the specificity of these reactions was obtained in the experiment shown in Fig. 2. The reduction of B6 T-cell cytotoxicity for Meth 4 cells by treatment with anti-Ly-1.2 serum was prevented by absorption with B6 thymocytes (phenotype Ly-1.2), but not by absorption with thymocytes from congenic B6/Ly-1.1 mice (1).

TABLE I

Effect of Pretreatment of Syngeneic Cytotoxic Cells with Anti-Thy-1, Anti-MSLA, and Anti-Ig Sera

Pretreatment of peritoneal lymphocytes*	Exp. 1		Exp. 2	
	CMC	Reduction of CMC	CMC	Reduction of CMC
	%	%	%	%
Test for Thy-1				
Diluent only	72		70	
Anti-Thy-1.1	76	Standard	73	Standard
Anti-Thy-1.2	4	95	11	85
Test for MSLA and Ig				
Diluent only	66		66	
NRS‡	54	Standard	56	Standard
Anti-MSLA	4	93	8	86
Anti-Fab	80	−48§	ND‖	
Anti-κ	ND‖		76	−36§

* With C; this applies to all other experiments.

‡ Normal rabbit serum.

§ A negative entry signifies an increase in CMC, attributable most probably to an increase in the relative proportion of T cells present (since the number of viable cells added to each well, after pretreatment, was adjusted to equal the number of viable control lymphocytes added).

‖ ND, not done.

TABLE II

Effect of Pretreatment of Syngeneic Cytotoxic Cells with Ly Antisera

Peritoneal lymphocytes	Pretreatment	Remaining Meth 4 target cells	CMC	Reduction of CMC
		cpm	%	%
Nonimmune	Diluent only	1,811	0	
Immune	Diluent only	515	72	
Immune	Anti-Ly-1.1	529	71	Standard
Immune	Anti-Ly-1.2	1,268	30	58
Immune	Anti-Ly-2.2	1,501	17	76
Immune	Anti-Ly-3.2	1,192	34	52

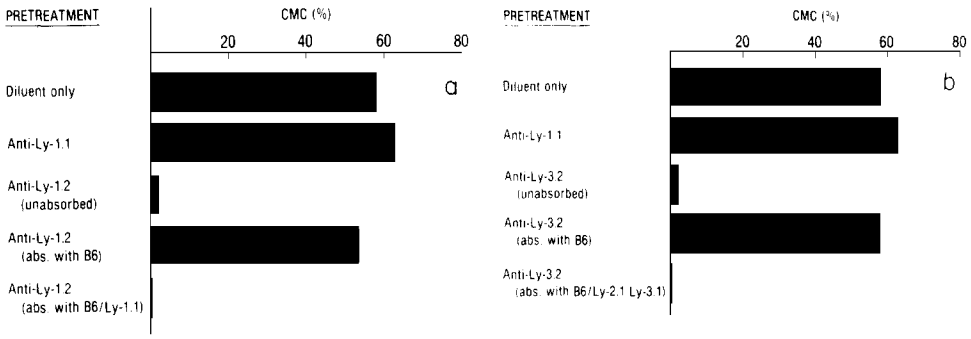

FIG. 2. Ly antisera, (*a*) unabsorbed, (*b*) absorbed (abs.) with B6 thymocytes, or (*c*) absorbed with thymocytes from Ly congenic B6 mice were used to pretreat effector cells. (2*a*) Ly-1.2 antiserum, which virtually abolished cytotoxicity when used unabsorbed, lost its inhibitory effect when absorbed with B6 thymocytes, but not with B6/Ly-1.1 thymocytes. (2*b*) The inhibitory activity of Ly-3.2 antiserum was removed with B6 thymocytes, but not with B6/Ly-2.1, 3.1 thymocytes. Anti-Ly-1.1 serum was used as an additional specificity control.

TABLE III

Failure to Reconstitute Syngeneic Cytotoxicity by Mixing Effector Cell Populations Pretreated with Either Anti-Ly-1.2 Serum or Anti-Ly-3.2 Serum

Peritoneal lymphocytes	Pretreatment	Remaining Meth 4 target cells	CMC	Reduction of CMC
		cpm	%	%
Nonimmune	Diluent only	1,710	0	
Immune	Diluent only	1,121	34	
Immune	Anti-Ly-1.1	857	50	Standard
Immune	Anti-Ly-1.2	1,790	0	100
Immune	Anti-Ly-3.2	1,845	0	100
Immune + Immune	Anti-Ly-1.2 + Anti-Ly-3.2	1,803	0	100

Immune lymphocytes were pretreated with one of the following: (*a*) diluent only, (*b*) anti-Ly-1.1 serum (standard), (*c*) anti-Ly-1.2 serum, or (*d*) anti-Ly-3.2 serum. Subsequently, 3×10^6 lymphocytes treated with anti-Ly-1.2 and C were mixed with 3×10^6 lymphocytes treated with anti-Ly-3.2 and C. The reconstituted cell population lacked cytotoxic activity.

Similarly, anti-Ly-3.2 was no longer effective in reducing lymphocyte cytotoxicity after absorption with B6 thymocytes (phenotype Ly-3.2), but retained that activity after absorption with B6/Ly-2.1,3.1.

These results suggested that Ly-1, as well as Ly-2/3 were represented on the surface of syngeneic cytotoxic cells. To determine whether some cytotoxic cells express Ly-1, and others Ly-2/3, or whether the phenotype of all cytotoxic cells was Ly-1,2,3, experiments of the type shown in Table III were undertaken. Effector cells were pretreated with either anti-Ly-1.2 or anti-Ly-3.2 serum, and then mixed in equal proportions to obtain a mixture that contained Ly-1 cells and Ly-2/3 cells, but not Ly-1,2,3 cells. This mixture (on line 6 in Table III) showed no cytotoxicity, demonstrating that lymphocyte cytotoxicity for syngeneic tumor cells is mediated by cells that carried both Ly-1 and Ly-2/3 on their surface. The results do not rule out the possibility that Ly-1,2,3 T cells are required to cooperate with other T cells in syngeneic cell killing or that an increase in the proportion of suppressor cells might account for the lack of cytotoxicity in the experiment with mixed effector cell populations.

Summary

Our present and previous findings may be summarized as follows: The phenotype of C57BL/6 (B6) cytotoxic cells for allogeneic target cells is Thy-1^+, Ly-1^-, Ly-$2/3^+$, MSLA$^+$, and Ig$^-$. The phenotype of B6 cytotoxic cells for syngeneic tumor cells is Thy-1^+, Ly-1^+, Ly-$2/3^+$, MSLA$^+$, and Ig$^-$. Thus, differences in Ly phenotype appear to be exhibited not only by cytotoxic T cells as opposed to helper T cells, but also within subcategories of cytotoxic T cells.
subcategories of cytotoxic T cells.

We thank Dr. Edward A. Boyse for helpful advice, and for providing antisera and mice of Ly congenic stocks used in these experiments. Mr. Willis V. Burton and Miss Joan M. Feld performed excellent technical work.

Received for publication 19 July 1976.

References

1. 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 the surface phenotype of T cells. *J. Exp. Med.* **141**:227.
2. Kisielow, P., J. Hirst, H. Shiku, P. C. L. Beverley, M. K. Hoffmann, E. A. Boyse, and H. F. Oettgen. 1975. Ly antigens: markers for functionally distinct sub-populations of thymus derived lymphocytes of the mouse. *Nature (Lond.)*. **253**:219.
3. Cantor, H., and E. A. Boyse. 1975. 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. *J. Exp. Med.* **141**:1376.
4. Shiku, H., M. A. Bean, L. J. Old, and H. F. Oettgen. 1975. Cytotoxic reactions of murine lymphoid cells studied with a [^{3}H]proline microcytotoxicity test. *J. Natl. Cancer Inst.* **54**:415.
5. Shen, F. W., E. A. Boyse, and H. Cantor. 1975. Preparation and use of Ly antisera. *Immunogenetics*. **2**:1.
6. Shiku, H., T. Takahashi, M. A. Bean, U. Hämmerling, H. F. Oettgen, and L. J. Old. 1976. Surface phenotype of non-adherent peritoneal cells effecting cell-mediated cytotoxicity *in vitro* for allogeneic and syngeneic murine sarcoma cells: *Isr. J. Med. Sci.* **12**:425.