

DIRECT EVIDENCE THAT NATURAL KILLER
CELLS IN NONIMMUNE SPLEEN CELL POPULATIONS
PREVENT TUMOR GROWTH IN VIVO*

BY M. KASAI, J. C. LECLERC,‡ L. McVAY-BOUDREAU, F. W. SHEN, AND H.
CANTOR§

From the Harvard Medical School, Farber Cancer Institute, Boston, Massachusetts 02115

Splenic lymphocytes from nonimmune mice (or blood lymphocytes from people) can lyse or damage a variety of target cells, notably malignant cells, *in vitro*. This reaction has been termed natural killer (NK) activity, because no overt immunization is required. NK activity is independent of antibody and complement and does not reflect conventional phagocytic activity. Numerous additional studies have sharply distinguished NK cells from characteristic T lymphocytes, B lymphocytes, and macrophages (1-8).

NK cells have received considerable attention because of their potential role in resistance to malignancy. Mice lacking a normal T-cell system probably do not develop an inordinately high incidence of spontaneous tumors (9, 10). The case for immunologic surveillance against neoplastic growth may therefore rest, in part, upon the participation of a non-T-cell population. This hypothetical cell population should be capable of destroying very small numbers of nascent tumor cells that might otherwise slip through the T-cell system. One candidate for this non-T-cell set is the NK-cell system, in view of experiments that indicate a positive correlation between *in vitro* NK-lytic activity and *in vivo* resistance to several lymphoid tumors (11-13).

Despite this association between NK function and resistance to tumor growth, it has not been possible to directly test whether NK cells can prevent *in vivo* tumor growth. Such tests depend upon the ability to obtain purified NK cells from heterogeneous cell populations. We have established that NK cells express both the NK-1- (14) and Ly5-surface components (15), and that antisera that is specific for the latter cell-surface component allows highly efficient positive selection of NK cells from a heterogeneous Thy1⁻ cell population. We demonstrate here that, unlike other sets of spleen cells from nonimmune mice, positively selected NK cells prevent *in vivo* growth of lymphomas.

Materials and Methods

Mice. B6, BALB/c, and A/SN mice were purchased from The Jackson Laboratories, Bar Harbor, Maine. B6-Ly5.2 congenic mice were produced by E. A. Boyse at the Sloan-Kettering Cancer Center, New York.

Tumor Cells. RL δ 1 is a BALB/c radiation-induced leukemia (16) and is carried *in vitro* in

* Supported by U. S. Public Health Service grants AI-13600, AI-12184, and CA-22131.

‡ Present address: Institut National de la Santé et de la Recherche Médicale, Hospital Cochin, Paris, France.

§ Scholar of the Leukemia Society of America.

our laboratory as described previously (14). YAC is a Moloney virus-induced lymphoma of A/J mice (2) and the ascites form is passaged *in vivo* in A/J mice in this laboratory.

Positive Selection of Ig⁺ Spleen Cells. The Ig⁺ fraction of spleen cells was eluted from Petri dishes coated with R α -mouse Fab, according to the method of Wysocki and Sato (17). After two sequential elutions, the remaining population was 96–100% Ig⁺ as judged by immunofluorescence.

Positive Selection of Ly5⁺ Cells or Thy1⁺ Cells. Spleen cells from B6, B6-Ly5.2, BALB/c, or A/J mice are passed through a nylon-wool column and treated with anti-Thy1.2 (final dilution 1:20) and a selected rabbit serum as a source of complement. These cells are washed three times and incubated for 30 min at 4°C with anti-Ly5.1 or normal mouse serum (NMS) (final dilution of 1:20) in the absence of complement. 5 ml of these cells (10⁷/ml) are added to Petri dishes (100 × 20-mm Falcon 3003, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnord, Calif.) coated with a monolayer of sheep erythrocytes (SRBC) conjugated to protein A. Coupling of SRBC to protein A was performed according to the method of Parrish and Hayward (18); protein A-SRBC monolayers were prepared according to the method of Stulting and Burke (19).

After a 15-min incubation of lymphocytes at 4°C, the plates are gently swirled and incubated an additional 15 min at 4°C. Unbound (nonadherent) cells are removed by gently aspirating the supernate with a Pasteur pipette. Bound cells are released from the monolayers after the addition of 2 ml of distilled water, for 15 s, followed by the addition of 2 ml of 2 N phosphat-buffered saline. This procedure lyses SRBC and permits recovery of the bound lymphocyte population. The recovered cell populations (unbound plus bound) represent \cong 80–95% of the starting (input) cell population.

Analysis of the Specificity of Positive Selection Using Ly5.1 or Thy1.2 Antiserum. Spleen cells from B6 (Ly5.1⁺) mice were passed through nylon wool columns, treated with anti-Thy1 + C, washed, and incubated with anti-Ly5.1 as described above. The nonadherent and adherent fractions of cells obtained after incubation on protein A-coated Petri dishes were incubated at 37°C for 3 h in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% FCS. This 3-h incubation procedure is required to eliminate any mouse immunoglobulin bound to lymphocytes during the positive selection procedure. After one additional wash, the cells were then incubated with either anti-Ly5.1 or NMS + C. Background lysis (after incubation with NMS + C) was 14–18%. Analysis of the input population (nylon-passed, anti-Thy1 + C-treated cells) indicated that this population contained \cong 15% Ly5.1⁺ cells. Analysis of the separated populations showed that the nonadherent cell population contained <5% Ly5.1⁺ cells, whereas the adherent fraction contained >80% (82–87%) Ly5.1⁺ cells. This separation was specific because (a) separation of cells coated with anti-Ly5.1 sera that had been absorbed with B6 spleen cells (equal volumes of packed cells and antisera at a 1:5 dilution for ½ h at 4°C) did not result in the enrichment of Ly5.1⁺ cells in the bound population; and (b) absorption of anti-Ly5.1 sera with B6-Ly5.2 cells had no effect upon the efficiency of positive selection. Similar analysis of the specificity of selection using Thy1.2 antisera indicated that the bound population (representing 30–34% of spleen cells) was 95–98% Thy1.2⁺.

Assay for In Vitro NK Activity. NK activity was determined according to a modification of a previously described protocol (14). 3 × 10⁴ ⁵¹Cr-labeled target cells were incubated in RPMI-1640 plus 10% fetal calf serum alone or together with different spleen cell populations at 37°C in a humidified atmosphere of 95% air, 5% CO₂. The amount of gamma radioactivity released from triplicate cultures after 5 h was determined. Cytotoxicity is expressed as percent specific lysis according to the following formula:

$$\text{percent lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release (freeze thaw)} - \text{spontaneous release}} \times 100.$$

Measurement of In Vivo Tumor Growth. 8 × 10⁴ RL δ 1 cells are inoculated (alone or with lymphoid cells) subcutaneously in 0.1 ml using a 28-gauge needle (Sherwood Medical Industries, Inc., DeLand, Fla.) beneath the shaved flanks of syngeneic BALB/c (400 rads) mice. The appearance of a white-capped bleb signifies accurate placement. 10⁴ YAC lymphoma cells were similarly inoculated into unirradiated syngeneic A/J mice. No growth signifies animals that did not develop tumor within 35 d after inoculation. Growth was invariably progressive until death of the host.

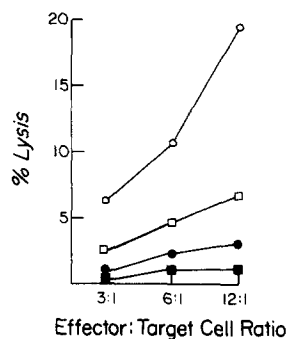


FIG. 1. Enrichment of in vitro NK activity after positive selection with anti-Ly5.1. Percent lysis of ^{51}Cr -labeled RL δ 1 cells after a 5-h incubation with unselected spleen cells (□); Ly5.1⁻ spleen cells (■); Thy1⁻Ig⁻Ly5⁺ cells (○); and Thy1⁻Ig⁻Ly5⁻ cells (●).

Results

Enrichment of In Vitro NK Activity after Positive Selection (Fig. 1). Spleen cell populations, depleted of Ly5⁺ cells, exerted virtually no detectable NK activity. By contrast, Ig⁻Thy1⁻ populations, containing $\cong 80\%$ Ly5⁺ cells after positive selection, exerted substantial NK lysis. This activity represented \cong a four- to six-fold enrichment compared to unselected spleen cells. Enhanced NK activity was a result of the enrichment of Ly5⁺ cells because NK activity was abolished after treatment of these cells with anti-Ly5.1 (but not anti-Ly5.2 or NMS) + C.

In Vivo Activity of Ly5⁺ NK Cells (Table I). Subcutaneous inoculation of 8×10^4 RL δ 1 cells into sublethally irradiated (400 rads) syngeneic BALB/c mice results in 100% tumor growth within 15 d. Subcutaneous inoculation of 10^4 YAC cells into unirradiated A/J mice results in 100% tumor growth within 16–18 d. Subcutaneous growth of either RL δ 1 or YAC tumor cells was not affected by co-inoculation of ($5\text{--}10 \times 10^6$) the following syngeneic (BALB/c or A/J, respectively) lymphocyte populations: (a) nonimmune spleen cells; (b) nylon-passed spleen cells; (c) Thy1⁺ spleen cells; (d) Ig⁺ spleen cells; and (e) Thy1⁻ spleen cells (after treatment with anti-Thy1 + C).

By contrast, spleen cells expressing the Ig⁻Thy1⁻Ly5⁺-surface phenotype (which account for <5% of the total spleen cell population) conferred virtually complete protection against the growth of either RL δ 1 or YAC tumor cells. This protective effect was a result of Ly5⁺ NK cells because no protective effects were exerted by equal numbers of viable Ig⁻Thy1⁻ cells that remained after treatment with anti-Ly5.1 + C.

Summary

Relatively large numbers of nonimmune spleen cells do not protect against the local growth of two lymphomas. However, this heterogeneous population of splenic lymphocytes contains a subset of cells that efficiently protects against in vivo tumor growth. This cell population (cell-surface phenotype Thy1.2⁻Ig⁻Ly5.1⁺) represents <5% of the spleen cell population and is responsible for in vitro NK-mediated lysis.

Although these studies clearly and directly demonstrate that Ly5⁺ NK cells selected from a heterogeneous lymphoid population from nonimmune mice can protect syngeneic mice against local in vivo growth of two different types of tumor cells (in

TABLE I
Ability of Different Lymphocyte Sets to Prevent Lymphoma Growth In Vivo

Spleen cell population	Cell number ($\times 10^6$)	Tumor incidence*	
		Host: BALB/c Tumor: RL δ 1	A/J YAC
A None	—	22/22	16/16
B Unselected	10	8/8	8/8
C Nylon-passed cells	5	14/15	15/15
D Thy1 ⁺ cells	5	8/8	8/8
E Ig ⁺ cells	5	16/16	16/16
F Thy1 ⁻ cells	5	19/22	22/22
G Thy1 ⁻ Ig ⁻ Ly5 ⁺ cells	2	1/12	0/12
H Group G treated with anti-Ly5.1 + C	2	7/8	7/8

* Results of three individual experiments.

contrast to other lymphocyte sets within the spleen), they do not directly bear upon the role of NK cells in immunosurveillance. They do indicate that highly enriched Ig⁻Thy1⁻Ly5⁺ cells, which account for virtually all in vitro NK activity, can retard tumor growth in vivo.

It is difficult to ascribe all anti-tumor surveillance activity to NK cells, because they probably do not recirculate freely throughout the various organ systems of the body. Perhaps NK cells may play a role in prevention of neoplastic growth within discrete anatomic compartments where there is rapid differentiation of stem cells to mature progeny (e.g., bone marrow, spleen, and portions of the gastrointestinal tract) and may normally act to regulate the growth and differentiation of non-neoplastic stem cells. Long-term observation of chimeric mice repopulated with bone marrow from congenic or mutant donors expressing very low or very high NK activity may help to answer these questions.

Received for publication 12 February 1979.

References

- Greenberg, A. H., and M. Greene. 1976. Non-adaptive rejection of small tumour inocula as a model of immune surveillance. *Nature (Lond.)* **264**:356.
- Kiessling, R., E. Klein, and H. Wigzell. 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* **5**:112.
- Kiessling, R., E. Klein, H. Pross, and H. Wigzell. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5**:117.
- Greenberg, A. M., and J. H. Playfair. 1974. Spontaneously arising cytotoxicity to the P-815-Y mastocytoma in NZB mice. *Clin. Exp. Immunol.* **16**:99.
- Sendo, F., T. Aoki, E. A. Boyse, and C. K. Buafo. 1975. Natural occurrence of lymphocytes showing cytotoxic activity to BALB/c radiation-induced leukemia RL δ 1 cells. *J. Natl. Cancer Inst.* **55**:603.
- Herberman, R. B., M. E. Nunn, and D. H. Lavrin. 1975. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer.* **16**:216, 1975.
- Herberman, R. B., M. E. Nunn, H. T. Holden, and D. H. Lavrin. 1975. Natural cytotoxic

- reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int. J. Cancer*. **16**:230.
8. Kiessling, R., G. Petranyi, G. Klein, and H. Wigzell. 1976. Non-T cell resistance against a mouse Moloney lymphoma. *Int. J. Cancer* **17**:1.
 9. Stutman, O. 1974. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. *Science (Wash. D. C.)*. **183**:534.
 10. Möller, G. 1976. The nude mouse vs. the hypothesis of immunologic surveillance. *Transplant. Rev.* **28**:1.
 11. Kiessling, R., G. Petranyi, K. Karre, M. Jondal, D. Tracey, and H. Wigzell. 1976. Killer cells: a functional comparison between natural, immune T-cell and antibody-dependent in vitro systems. *J. Exp. Med.* **143**:772.
 12. Petranyi, G., R. Kiessling, S. Povey, G. Klein, L. Herzenberg, and H. Wigzell. 1976. The genetic control of natural killer cell activity and its association with in vivo resistance against a Moloney lymphoma isograft. *Immunogenetics*. **3**:15.
 13. Haller, O., M. Hansson, R. Kiessling, and H. Wigzell. 1977. Nonconventional natural killer cells may play a decisive role in providing resistance against syngeneic tumor cells in vivo. *Nature (Lond.)*. **270**:609.
 14. Glimcher, L., F. W. Shen, and H. Cantor. 1977. Identification of a cell-surface antigen expressed on the natural killer cell. *J. Exp. Med.* **145**:1.
 15. Kasai, M., J. C. Leclerc, F. W. Shen, and H. Cantor, 1979. Identification of Ly5 on the surface of "natural killer" cells in normal and athymic inbred mouse strains. *Immunogenetics*. In press.
 16. Sato, H., E. A. Boyse, T. Aoki, C. Iritani, and L. J. Old. 1973. Leukemia-associated transplantation antigens related to murine leukemia virus. *J. Exp. Med.* **138**:593.
 17. Wysocki, L., and V. L. Sato. 1978. Panning for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 2844.
 18. Parrish, C. R., and J. A. Hayward. 1974. The lymphocyte surface. I. Relation between Fc receptors, C'3 receptors and surface immunoglobulin. *Proc. R. Soc. Lond. B Biol. Sci.* **B187**: 47.
 19. Stulting, R. D., and G. Burke. 1973. Nature of lymphocyte-tumor interaction. *J. Exp. Med.* **137**:932.