TUMOR NECROSIS SERUM INDUCES A SEROLOGICALLY DISTINCT POPULATION OF NK CELLS*

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The injection of bacterial lipopolysaccharide (LPS)[1] in mice causes the release of biological mediators into the serum. High concentrations of factors with various biological effects are found in the serum of mice that were treated with Bacille bilié de Calmette-Guérin (BCG) or *Corynebacterium parvum* before injection with LPS. Serum of mice so-treated is called tumor necrosis serum (TNS), because it causes acute partial necrosis and subsequent regression of syngeneic tumor transplants in mice (1). In addition to the anti-tumor effect, TNS influences immunological functions: it contains factors that replace helper T cells in the humoral immune response (2) and that induce phenotypic and functional differentiation of B lymphocytes (3).

In this report, we investigate the effect of TNS on the generation of cytotoxic cells in vitro. We show that two serologically distinct types of cytotoxic cells are generated in murine spleen-cell cultures in the absence of specific stimulus. One type arises in the presence of TNS and expresses the cell-surface antigen, Qa5, which is controlled by the *Q* region of chromosome 17 (4). This cell does not express the T-cell surface marker Thy-1, in contrast to a second type that arises spontaneously in tissue culture. We refer to both types of nonspecific cytotoxic cells as natural killer (NK) cells.

NK cells appear in murine spleens at ≅3 wk of age and become undetectable ≅3 mo later (5, 6). Serological studies indicate the expression of Thy-1 (7), Ly-5 (8), and NK (9) cell-surface antigens on NK-cell populations. A number of reagents increase NK activity in young animals and induce reoccurrence of NK cells in older animals. Among these reagents are LPS (10), BCG (11), and *C. parvum* (12); substances that are prerequisites for the release of biologically active mediators in TNS (1).

Materials and Methods

Mice. C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c·nu mice were provided by Mr. C. Reeder, of the National Institutes of Health, Bethesda, Md.

Reagents. TNS was kindly provided by E. Richards, of the Memorial Sloan-Kettering Cancer Center, New York. It was prepared as described previously (1) by exsanguinating *C.*

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[1] *Abbreviations used in this paper:* BCG, Bacille bilié de Calmette-Guérin; C, complement; LPS, bacterial lipopolysaccharide; NK, natural killer; NMS, normal mouse serum; TNS, tumor necrosis serum.

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parvum-treated mice (1 mg/mouse) 1 h after injection of LPS (20 μg/mouse). Mouse interferon (10^6 U/0.4 mg) was obtained from Dr. William Stewart, Jr. of the Memorial Sloan-Kettering Cancer Center.

Cell Cultures. Murine spleen cells were cultured at a cell concentration of 5×10^6 cells/ml according to the method of Mishell and Dutton (13) except that 2-mercaptoethanol (5×10^{-5} M) was added to the medium.

Sera. Monoclonal anti-Thy-1.2 antibody (clone 6/68) and anti-Qa5 antibody (clone B16/167) were prepared as described previously (4). For the removal of cells expressing Thy-1 or Qa5, spleen cells were incubated in minimum essential medium (containing 2% fetal calf serum) for 45 min at 37°C in presence of antibody (1:100) and complement (C) (nontoxic rabbit serum, diluted 1:10). Cells were washed twice, and readjusted to 5×10^6 living cells before culture. In experiments aimed at determining the phenotype of effector cells, cultured spleen cells were treated similarly with antibody and C; however, experimental populations (treated with antibody) were adjusted to the volume of control cells (treated with normal mouse serum [NMS]) containing 5×10^6 cells/ml.

Cytotoxicity Test. Target cells (RLδ1, BALB/c-derived, radiation-induced leukemia cells, maintained in tissue culture) were incubated with ^{51}Cr (100 μC/7×10^6 cells in 0.5 ml culture medium) for 60 min, washed twice, and adjusted to 10^5 cells/ml. 0.1 ml of ^{51}Cr-labeled target cells were mixed with 0.1 ml effector cells in ratios of 200:1, 40:1, 8:1, and 1.6:1. Cell mixtures were incubated for 6 h at 37°C with a gas mixture of 7% O_2, 10% CO_2, and 83% N_2. Released ^{51}Cr activity was determined using a Titerteck supernate collection system (Flow Laboratories, Inc., Rockville, Md.) and a Packard gamma scintillation counter (Packard Instrument Co., Downers Grove, Ill.). The percentage of cytotoxicity was calculated as described previously (5).

Results and Discussion

Spleens of athymic BALB/c·nu mice contain NK cells (5, 14) (Fig. 1). NK cells lost, or at best maintained, cytotoxic activity in tissue culture. Addition of TNS to the culture medium induced marked NK activity in culture of BALB/c·nu spleen cells (Fig. 1). NK-cell activity in TNS-treated spleen-cell cultures reached a peak between day 1 and 2. Spleen cells from mice with normal thymic function generated, in the presence of TNS, two peaks of cytotoxic activity, one peak between day 1 and day 2, and a second peak between day 4 and day 5. In the absence of TNS, normal spleen cells generated only the later peak of NK-cell activity (Fig. 2 A). The failure of BALB/c·nu spleen cells to generate this second peak of NK-cell activity indicated that T cells were required for its generation. To verify this conclusion, we treated C57BL/6 spleen cells with anti-Thy-1.2 antibody and C and found that spleen cells thus depleted of T cells generate early NK-cell activity in response to stimulation with TNS, but not the second peak of NK-cell activity (Fig. 2 B). In screening a series of monoclonal antibodies against distinct lymphocyte cell-surface components, we found one antibody (anti-Qa5) which lysed spleen cells that were required for the generation of the early NK-cell activity (induced by TNS) but which did not abrogate the generation of later NK cells that arise independently of TNS (Fig. 2 C). Taken together, the data in Fig. 2 show that two populations of NK cells can be distinguished in murine spleens based on the requirements for their generation. One type depends on cells that express the Qa5 surface marker and requires TNS for its activation. The other type requires T cells for its generation and arises in the absence of TNS.

The phenotype of effector cells was determined in standard negative immunoselective procedures using cells collected from cultures at the early (day 1) or the late (day 4) peak of NK-cell activity (Table I). Cells were obtained from spleen cultures that were stimulated with TNS. NK activity from spleen cells collected on day 1 was abrogated by treatment with anti-Qa5 antibody and C, but not by treatment with

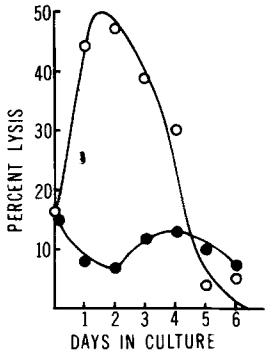

FIG. 1. Induction of nonspecific killer cells in cultures of BALB/c·nu spleen cells. BALB/c·nu cell suspensions (5×10^6 cells/ml) were cultured in the presence (○) or absence (●) of TNS (5 μl/ml). Cells were harvested at times indicated and incubated with ^{51}Cr-labeled target cells in a ratio of 100 spleen cells to 1 target cell. Each value was obtained from triplicate cultures, and the data shown represent the mean of three experiments. NMS or serum from mice treated with LPS or *C. parvum* alone did not produce NK-cell activity (not shown).

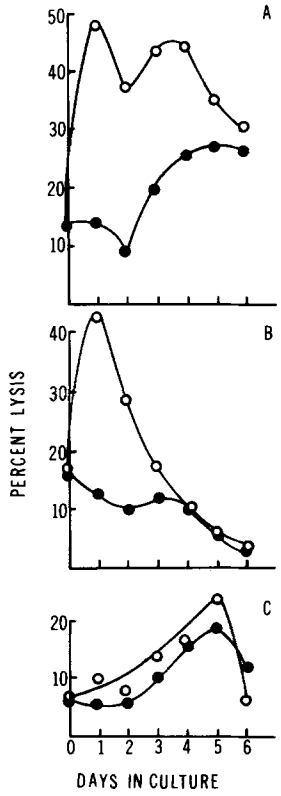

FIG. 2. Generation of NK cells in culture of C57BL/6 spleen cells after treatment with anti-Thy-1.2 antibody or anti-Qa5 antibody. Spleen cells were cultured in the absence (●) or presence (○) of TNS for the indicated time intervals and assayed for cytotoxic activity. Before culture, spleen cells were treated with: (A) NMS (1:100) and C (rabbit serum 1:10); (B) monoclonal anti-Thy-1.2 antibody (1:100) and C; (c) monoclonal anti-Qa5 antibody (1:100) and C. Each point represents the mean of four experiments.

TABLE I

Phenotype of In Vitro-Induced NK Cells

NK cells collected at	Percentage of cytotoxicity after treatment of cells with:		
	NMS + C	Anti-Thy-1.2 + C	Anti-Qa5 + C
		%	
Early peak (day 1)	41 ± 9	27 ± 9	4 ± 3
Late peak (day 4)	32 ± 11	16 ± 2	28 ± 5

C57BL/6 spleen cells were cultured with TNS and harvested on day 1 or day 4 of culture. Before cytotoxic activity was determined, cells were incubated with either NMS (1:100), monoclonal anti-Thy-1.2 antibody (1:100), or monoclonal anti-Qa5 antibody (1:100) and C (rabbit serum 1:10) for 45 min at 37°C. The data represent the mean ±SD of six experiments.

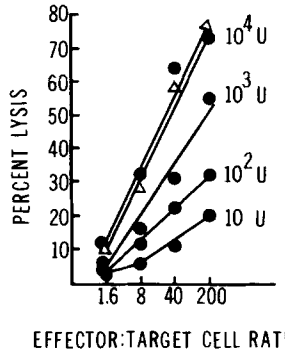

FIG. 3. NK-cell activity induced by interferon. BALB/c-nu spleen cells were cultured in the presence of TNS (Δ) (5 μl/ml) or interferon (●) (U/ml as indicated). Spleen cells were harvested after 24 h in culture. Each value represents the mean of triplicate cultures from one experiment.

anti-Thy-1 antibody and C. In contrast, spleen cells collected on day 4 did not lose their cytotoxic activity after treatment with anti-Qa5 antibody, but showed an increased sensitivity to treatment with anti-Thy-1 antibody. Treatment of effector cells with anti-Thy-1 antibody did not, however, discriminate between the two types of NK cells as decisively as treatment of spleen cells before culture. It has been our experience in the past that cultured T lymphocytes are more resistant to lysis with anti-Thy-1 serum and C than fresh spleen T cells. Therefore, our failure to completely remove in vitro-induced cytotoxic cells by treatment with anti-Thy-1 antibody and C does not necessarily indicate that these cytotoxic cells are not T cells.

Initial attempts were made to identify the component in TNS that is involved in the activation of NK cells in vitro. TNS contains substantial amounts of interferon (1). Because this agent has been shown to activate NK cells (15, 16), we considered the possibility that it is the interferon in TNS that causes generation of NK cells in vitro. This view is supported by experiments in which interferon, like TNS, generated NK-cell activity of cultures of murine spleen cells within the first 24 h. In this response, 10^3 U of interferon were equivalent to 5 μl of TNS (Fig. 3). Interferon-induced NK cells expressed, like TNS-induced NK cells, Qa5 surface antigen (data not shown).

Our data indicate the existence of two types of cytotoxic cells arising in tissue culture in the absence of specific antigenic stimulation. We refer to both types of nonspecific cytotoxic cells as NK cells. We believe that our results will make it necessary to define the class of natural killer cells more precisely with regard to their lineage derivations. The present study provides some criteria for a definition in terms of phenotype and requirement for their activation.

The physiological role played by NK cells is not known. It has been speculated that NK cells are involved in immune surveillance (17, 18). If the concept of immune surveillance (19, 20) is valid, it must also apply to mice with defective thymic functions (BALB/c·nu mice), because the tumor incidence in these animals is not increased (21, 22). The observation of an NK cell type that arises in spleen cell populations from athymic as well as normal mice and that is distinct from thymus-dependent cytotoxic cells is, therefore, significant in the context of the immune surveillance concept.

Summary

Murine spleen cells generate nonspecific cytotoxic cells, referred to as natural killer (NK) cells, within 4 d of incubation in Mishell-Dutton cultures. This NK cell type does not arise in cultures of BALB/c·nu spleen cells or in cultures of T-cell depleted C57BL/6 spleen cells, indicating that its activation depends on T cells. Another type of NK cells is induced by tumor necrosis serum in murine spleen-cell cultures. It arises within 24 h and its activation does not depend on T cells. This cell type (and its precursor) expresses the recently discovered cell-surface marker Qa5 (controlled by the *Q* region of chromosome 17) that distinguishes this NK cell from the NK cell that depends for its activation on thymic function. $Qa5^+$ NK cells are also induced by interferon.

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