IDENTIFICATION OF A CELL-SURFACE ANTIGEN SELECTIVELY EXPRESSED ON THE NATURAL KILLER CELL*

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Spleen cells from certain unimmunized mouse strains can kill a variety of murine leukemia virus ($MuLV^+$) syngeneic or allogeneic tumor cells. This type of 'natural killer' (NK)[1] activity has now been reported in many in vitro systems (1–15). Several studies, mainly by Kiessling and his colleagues (9–15), have suggested (*a*) although NK cells are small lymphocytes, they cannot be classified as mature T, B, or monocyte-macrophage cells using standard cell fractionation procedures, (*b*) NK cells apparently do not bear Fc receptors on their surface and do not mediate antibody-dependent cellular cytotoxicity, (*c*) the presence of NK activity is controlled in part by genes within the *H-2* complex, and (*d*) there is a correlation between the presence of in vitro NK activity and in vivo resistance to many lymphoid tumors.

Despite this association between NK activity and resistance to tumor growth, it has not been possible to directly test their role in the prevention of tumor growth in vivo or to establish the developmental relationship of NK cells to other lymphoid subpopulations. These tests require the ability to selectively deplete or purify this lymphocyte subclass from a heterogeneous cell population. We describe here the serologic definition of a cell surface component which is selectively expressed on the surface of NK cells active against an $MuLV^+$ tumor.

Materials and Methods

Mice. C57BL/6 (B6) mice (Ly phenotype 1.2,2.2,3.2; $H\text{-}2^b$) and BALB/c mice (Ly phenotype 1.2,2.2,3.2; $H\text{-}2^d$) were obtained from The Jackson Laboratory, Bar Harbor, Maine and the congenic line, B6/Ly1.1, from Dr. E. A. Boyse. NZB, C3H/Bi, CBA/T6, BALB/c, C3H/An, DBA/2, and (BALB/c × B6)F_1 ($H\text{-}2^{bd}$) were bred in our animal facilities from breeders supplied by The Jackson Laboratory.

Antisera. The production and use of anti-Ly1.2 (C3H anti-CE thymocyte antiserum), anti-Ly2.2, and anti-Thy1.2 have been described previously (16). The Ly antisera were diluted 1:30 and absorbed twice with approximately 120×10^6 C3H thymus cells/ml to remove autoantibody. Anti-Thy1.2 was raised in (A/Thy1.1 × AKR)F_1 mice immunized with A-strain leukemic cells (ASLI); this serum had a titer of 1:200 against LN cells and was used at a final dilution of 1:40.

Tumor Cell Line. The tumor used as target cell was the BALB/c ($H\text{-}2^d$) RL♂1 radiation-

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[1] *Abbreviations used in this paper:* ASLI, A-strain leukemic cells; B6, C57BL/6; MULV, murine leukemia virus; NK, natural killer; NMS, normal mouse serum; α, anti.

induced leukemia obtained from Dr. E. A. Boyse. These cells carry a strong MuLV-associated antigen, X.1, on their cell surface (17). An in vitro line of RL♂1 cells was established in our laboratory; the cells were grown in suspension in L15 medium supplemented with 15% tryptose phosphate broth and 15% fetal calf serum and used as targets directly after harvesting. The P815 DBA/2 (*H-2*^{d}) mastocytoma were also used after similar in vitro passage.

Enrichment of Spleen T Cells by Nylon Wool Columns. Nylon wool columns were used as described previously (18), 80–85% of the effluent cells were Ig^-Thy1^+, and 1–6% were Ig^+Thy1^-.

Cytotoxicity Assay. We used the ^{51}Cr release method of Brunner et al. (19) as modified by Canty and Wunderlich (20). Appropriate numbers of attacking cells were mixed with 5×10^4 ^{51}Cr-labeled target cells and incubated 3½–4 h in an atmosphere of 7% CO_2. Cytotoxic activity for each test cell population was determined by the amount of ^{51}Cr released from triplicate cultures of target cells during incubation compared with medium alone (control). Data were calculated by the following formula:

$$\% \text{ lysis} = \frac{\text{cpm (test population)} - \text{cpm (control)}}{\text{cpm maximum release (freeze-thaw)}} \times 100$$

Standard errors for each triplicate culture were invariably less than 20%.

Results

Spontaneous Cytotoxicity of B6 and NZB Lymphoid Cells to the RL♂1 Tumor (Table I). B6 and NZB spleen cells express 'spontaneous' cytotoxicity against the RL♂1 ($MuLV^+$) but not against P815 ($MuLV^-$). Levels of spleen cell cytotoxicity from B6 mice varied from experiment to experiment, ranging from 15–40% at a 100:1 attacker/target ratio and averaging about 20–30%. NK activity was consistently slightly higher in the NZB (25–40% lysis), a strain known to harbor MuLV.

Various lymphoid organs in the B6 and NZB were analyzed for NK activity. These results are in agreement with previous findings (13): (*a*) spleen cells are most active; (*b*) lymph node and bone marrow cells are significantly less active; and (*c*) thymus cells have no activity.

Surface Markers of the NK Cell

THE NK CELL IS NEITHER A T NOR A B LYMPHOCYTE (FIG. 1). Spleen cells from NZB and B6 mice that had passed through a nylon wool column (to deplete Ig^+ cells see Materials and Methods) expressed substantially increased NK activity compared with unpassed cells. Treatment of spleen cells with anti-Thy1 + complement (C) also did not reduce NK activity compared with normal mouse serum (NMS) + C-treated control spleen cells. These data indicate that the NK cell is not a typical mature T or B cell.

NK CELLS EXPRESS A CELL SURFACE ANTIGEN RECOGNIZED BY C3H α-CE ('ANTI-LY1.2') ANTISERUM (FIG. 2). To determine whether the NK cells expressed Ly antigens, we treated nylon column-passed spleen cells with Ly sera + C. Treatment with either anti-Ly2.2 or anti-Thy1.2 + C had no affect on NK activity. However, treatment with C3H anti-CE antiserum (anti-Ly1.2) abolished NK activity (Fig. 2).

We were surprised by these results. Ly1 antigens are thought to be expressed exclusively on T cells, and the NK cell bears no other T-cell markers (e.g., Thy1, Ly2). Therefore, we suspected that the C3H anti-CE antiserum contained antibodies to determinants other than Ly1.2 which are expressed on NK cells. To test this hypothesis, we examined the effects of C3H αCE serum + C upon

TABLE I
Tissue Distribution of NK Activity against RL♂1 Tumor Target Cells

Donor strain	Percent lysis (100:1 A/T ratio)*	
	RL♂	P815‡
B6		
Spleen	22 ± 2	0
LN	12 ± 1.5	0
BM	10 ± 3	0
Thymus	0 ± 0.5	0
NZB		
Spleen	34 ± 2	0
LN	14 ± 3	0
BM	10 ± 2.5	0
Thymus	4 ± 1.5	0

* See Materials and Methods for details of assay.

‡ In most cases net ^{51}Cr release from mixtures of untreated cell populations with P815 was less than that released from cultures containing P815 + medium.

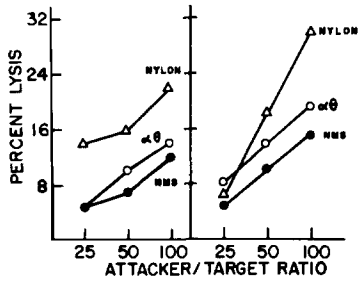

FIG. 1. The effect of passage through a nylon column or treatment with anti-Thy1 on NK activity. NK activity of spleen cells after (*a*) passage through a nylon column (△), (*b*) treatment with anti-Thy1 plus C (○), and (*c*) treatment with NMS + C (●). B6 cell donors (left panel), NZB donors (right panel).

spleen cells from a congenic pair of B6 mouse strains differing only at the Ly1 locus, B6/Ly1.1, and B6(Ly1.2$^+$). NK activity of spleen cells from both Ly1.1$^+$ and Ly1.2$^+$ congenic B6 strains were equally sensitive to the C3H anti-CE (anti-Ly1.2) antiserum (Fig. 3). Hence anti-NK antibody in this serum was not directed at the Ly1.2 antigen. To confirm this point, C3H αCE antisera were absorbed with either B6 or B6/Ly1.1 spleen cells; both absorptions removed αNK activity (Fig. 4).

Absorption of C3HαCE Sera to Produce αNK Sera (Fig. 5). We then absorbed C3H anti-CE antiserum with BALB/c thymus and spleen cells (which are NK$^-$ and Ly1.2$^+$). This absorption removed all anti-Ly1.2 activity, as tested by lysis of ^{51}Cr-labeled B6 lymph node cells, but left anti-NK activity intact. This absorbed antiserum, which lysed less than 5% of a whole spleen cell population, therefore reacted with a surface component selectively expressed on NK cells. No loss of NK activity was noted after further absorption of αNK sera

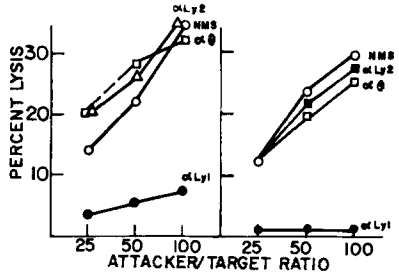

FIG. 2. NK activity of nylon wool passed spleen cells treated with Ly antisera and anti-Thy1. The effect of NMS + C alone (○), anti-Thy1 + C (□), anti-Ly2 + C (△), C3H anti-CE + C (anti-Ly1.2) (●) upon the NK activity of spleen cells from NZB donors (left panel) and B6 donors (right panel) is shown.

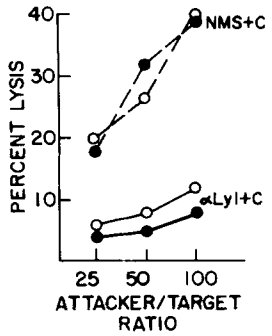

FIG. 3. The NK antigen is distinct from Ly1.2. NK activity of B6 spleen cells (●) and B6/Ly1.1 congenic spleen cells (○) after treatment with C3H anti-CE antiserum (anti-Ly1.2) or NMS. C3HαCE antiserum virtually eliminated NK activity from spleen cells of both Ly congenic strains.

(1:30) with equal volumes of brain cells, kidney cells, or fibroblasts (data not shown).

Strain Distribution of αRL♂1 NK Activity and Susceptibility to αNK Antisera (Table II). Spleen cells from a variety of inbred mouse strains were tested for spontaneous cytolytic activity against RL♂1 target cells. Cells from BALB/c mice and DBA/2 mice did not express significant NK activity. Spleen cells from B6, (BALB/c × B6)F_1, and NZB mice produced substantial NK activity; this activity was eliminated after treatment with C3H αCE sera. CBA/T6 and C3H/Bi spleen cells also expressed significant levels of spontaneous lytic activity; this activity was resistant to treatment with C3H αCE sera + C.

Production of Specific αNK Antiserum in (C3H × BALB/c) F_1 Mice Immunized to CE Spleen Cells (Table III). To produce sera that would not require absorption with BALB/c cells to eliminate αLy1.2 activity, we immunized (C3H × BALB/c)F_1 mice with CE spleen cells according to a protocol described previously (16). This serum did not contain αLy1.2 activity (BALB/c Ly phenotype-Ly1.2, 2.2, 3.2), but retained anti-NK activity. Treatment of B6 spleen cells with this serum + C (*a*) did not result in detectable lysis above that seen with NMS + C, but (*b*) virtually eliminated all spontaneous lysis vs. RL♂1 targets. By contrast the sera had no effect on either B6 T-killer activity or plaque-forming cell activity (or T_H activity, data not shown). Thus insertion of the BALB/c

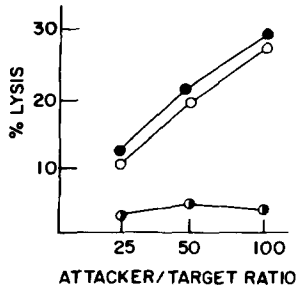

FIG. 4. Removal of anti-NK activity after absorption of anti-CE serum with spleen cells from either B6 or B6/Ly1.1 spleen cells. NK activity of nylon passed spleen cells treated with (*a*) C3H anti-CE serum that had been absorbed with 10^8 B6 spleen cells/ml, final dilution 1:40 (○), (*b*) C3H anti-CE absorbed with 10^8 B6/Ly1.1 spleen cells/ml, final dilution 1:40 (●), or (*c*) unabsorbed C3H anti-CE serum, final dilution 1:40 (◑).

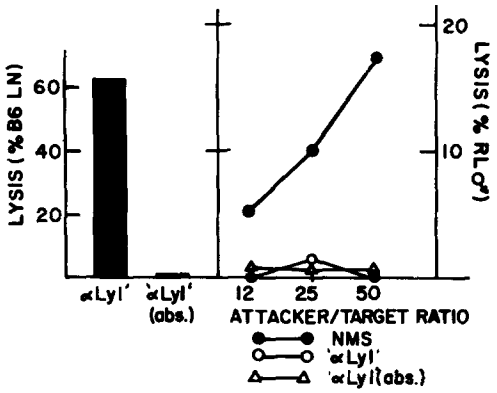

FIG. 5. Removal of αLy1.2 activity and retention of NK activity of C3HαCE antisera. C3H anti-CE antiserum was absorbed with BALB/c thymocytes plus spleen cells (2.5×10^8 spleen cells + 2.5×10^8 thymocytes/ml of C3H αCE, final dilution 1:30). The resulting serum did not lyse significant numbers of B6 LN-T cells (left panel), but retained αNK activity (right panel).

genotype into the host immunized with CE cells results in the production of an antiserum that reacts selectively with NK cells in the B6 mouse.

Discussion

Much recent immunologic research has focused on subclassifying cells of the immune system. Lymphoid cells have been divided on the basis of cell-surface markers into T cells, B cells, and 'null' cells involved in antibody-dependent cell-mediated cytotoxicity. T cells have been further subdivided into three major subclasses on the basis of selective expression of Ly gene products: helper cells, cytotoxic/suppressor cells, and immature cells whose immunoregulatory function is just beginning to be defined (21).

NK cells can specifically damage certain types of tumor cells which express MuLV-associated antigens, and this activity has been correlated with in vivo resistance to these tumors (12). However, it has not yet been possible to identify specific cell-surface markers on NK cells and thus directly test their role in the

TABLE II
Strain Distribution of NK Activity and Susceptibility to α NK Sera

Donor strain* (spleen cells)	H-2 haplotype	Percent lysis of RL♂ target‡		NK agn+
		NMS + C	αNK + C	
B6	*b*	29 ± 3	0	+
BALB/c	*d*	0	0	
(BALB/c × B6)F_1	*bd*	14 ± 1	3 ± 1	+
NZB	*d*	36 ± 2	3 ± 1	+
DBA/2	*d*	3 ± 1	5 ± 1	
CBA/T6	*k*	21 ± 1	19 ± 1.5	−
C3H/An	*k*	6 ± 1	6 ± 1.5	−
C3H/Bi	*k*	11 ± 2	10 ± 1.5	−

* Donors 4–8 wk of age.
‡ 60:1 attacker target ratio; see Materials and Methods for details of procedures.

TABLE III
The Effect of (C3H × BALB/c)F_1 αCE Sera Upon T-Killer Activity, PFC Activity, and NK Activity of B6 Spleen Cells

SRBC-PFC (10^6 cells)*		T-killer activity (percent lysis vs. P815‡)			NK activity (percent lysis vs. RL♂1§)		
NMS + C	αNK + C	A/T ratio	NMS + C	αNK + C	A/T ratio	NMS + C	αNK + C
390	420	10	44 ± 2	47 ± 4	60	36 + 3.5	4 ± 0.5
		5	31 ± 3.5	35 ± 1	30	28 ± 1	3 ± 0.5
		2.5	18 ± 1	21 ± 2	15	19 ± 2	3 ± 1

* Spleen cells from three B6 mice immunized with 10^7 SRBC six days earlier were treated with (C3H × BALB)F_1 αCE sera (final dilution 1:20) or NMS + RC just before testing for αSRBC PFC according to a technique described previously (22).
‡ Spleen cells from B6 mice given 3 × 10^7 (BALB × B6)F_1 spleen cells 9 days previously were treated with (C3H × BALB/c)F_1 αCE sera (final dilution 1:20) or NMS + RC, and then tested for cytolytic activity against ^{51}Cr-labeled P815 in a 4-h assay.
§ Spleen cells from nonimmune B6 mice were treated with (C3H × BALB/c)F_1 αCE sera (final dilution 1:20) or NMS + RC just before test for spontaneous lysis of RL♂1 cells (see Materials and Methods for protocol).

prevention of tumors in vivo. Although NK cells have the morphology of small lymphocytes, various cell fractionation procedures have indicated that these cells are not typical T cells, B cells, Fc receptor+ null cells, or monocyte-macrophages (13–15).

Our experiments confirm previous findings that NK cells are Thy1− and Ig− (13–15). In addition, we have identified a cell-surface antigen that is selectively expressed on NK cells. This surface component is recognized by C3H anti-CE thymocyte antiserum ('α Ly1.2'), but is distinct from the Ly1.2 surface antigen. We have produced an antiserum that reacts with NK cells but does not react with mature T or B cells by absorbing C3H anti-CE antiserum with cells from NK−, Ly1.2+ BALB/c mice. This absorption step was circumvented by inserting the BALB/c genotype into the recipient immunized to CE cells, i.e., (C3H × BALB/c) αCE antiserum. The resultant antiserum, 'αNK', killed <5% of B6 spleen cells, but selectively eliminated NK activity.

Our experiments used relatively high attacker:target ratios in order to demonstrate significant NK activity. It is likely that these high ratios were required because NK cells represent less than 5% of the whole spleen cell population, and therefore these studies reflect true NK cell/target ratios of about 1:1–1:5. These considerations indicate that the NK population may exert powerful cytolytic effects on appropriate tumor targets.

The C3H anti-CE thymocyte antiserum used in the first part of these experiments was made by injecting CE thymus cell suspensions into C3H mice. Since thymocytes do not express NK activity, it is somewhat surprising that this antiserum contained anti-NK antibody. There are several possible explanations: (*a*) NK cells may be present in CE thymus, albeit in very small numbers. The repeated injections of large numbers of CE thymocytes ($2–5 \times 10^7$/wk, 10–15 wk) necessary to produce αLy1.2 antiserum might also be sufficient to raise antibody against NK cells even though our functional assay does not detect significant NK activity. (*b*) The NK antigen in the thymus preparations may reflect the presence of contaminating NK cells from nonthymus tissue, including parathymic lymph node cells or circulating blood lymphocytes. (*c*) The thymus may contain NK cell precursors which bear the NK antigen, but do not yet express NK cell activity. (*d*) The existence of mature NK cells in thymus may be masked by thymic suppressor cells. Our data does not help select among these possibilities. Nonetheless, for whatever reason, C3H αCE thymocyte serum contains high titers of αNK antibodies.

Inasmuch as C3H mice produce anti-NK antibody, which eliminates NK activity from spleen cells of at least two strains, B6 and NZB, but does not affect NK activity of spleen cells from C3H/Bi or CBA/T6 mice, it is likely that the NK alloantigen is expressed either as two or more alleles including 'NK-C3H' and 'NK-B6' or possibly as a single allelic product that is not expressed by C3H or CBA mice. In either case, the present studies demonstrate that at least some mouse strains possess NK cells that can be selectively eliminated by the sera described in this report. The fact that the NK antigen is not expressed on the surface of mature T cells, B cells, kidney cells, or brain cells suggests that it falls into an increasingly useful category of cell-surface differentiation components that are selectively expressed on cells at particular phases of their differentiative history. The contribution of the NK cell-surface component defined in these studies to NK activity has not been extensively investigated, although preliminary experiments indicate the presence of αNK sera does not inhibit NK cell-mediated lysis.

The biologic significance of the NK cell is presently incompletely understood. Previous work has shown that (*a*) there is a correlation between in vitro NK activity and in vivo tumor resistance (12), and (*b*) there is a low incidence of RNA tumors in nude (congenital athymic) mice which have high NK activity (13, 14). For these reasons, it is possible that the NK cell may play a role in immunosurveillance against tumors. Selective removal of NK cells from spleen and bone marrow cells before adoptive transfer to irradiated hosts is now possible and will permit direct testing of this hypothesis.

Summary

We have studied the cell-surface phenotype of natural killer (NK) cells of NZB and B6 mice which react to an MuLV$^+$ lymphoid tumor. (*a*) NK cells do not express Thy1, Ly2, or Ig surface markers. (*b*) NK cells express an antigen recognized by C3H anti-CE antiserum ('anti-Ly1.2 antiserum'). Inasmuch as NK activity of spleen cells from B6 and B6/Ly1.1 congenic strains were both equally sensitive to C3H anti-CE antiserum, the NK antigen is distinct from Ly1.2. This point was confirmed by the observation that αNK activity was

removed by absorption of C3H anti-CE antiserum with spleen cells from either B6 or B6/Ly1.1 congenic strains.

Absorption of C3H αCE serum with BALB/c thymocytes and spleen cells (which are $Ly1.2^+NK^-$) removed anti-Ly1.2 activity and left anti-NK activity intact. This absorption step could be circumvented by inserting the BALB/c genotype into the recipient immunized to CE cells (i.e., (C3H × BALB/c)F_1 αCE spleen cells). This antiserum, provisionally termed 'anti-NK', defines a new subclass of lymphocytes which may play a central role in the immunosurveillance against tumors.

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