Natural Killer Cells

In Vitro and *In Vivo*

Christopher S. Henney, PhD, DSc, Daniel Tracey, PhD,
Jeannine M. Durdik, BS, and Gary Klimpel, PhD

A nonadherent, nonphagocytic mouse cell found in lymphoid organelles, but lacking characteristic surface markers of mature lymphocytes, is capable of lysing a wide spectrum of tumor cells but shows little cytolytic activity toward normal cells. This cytotoxic cell, termed a "natural killer" (NK) cell, shows a marked capacity to lyse lymphomas (syngeneic, allogeneic, or even xenogeneic) to the effector cell source. Its activity is inhibited by a variety of pharmacologic agents, eg, cytochalasins, cAMP-"active" drugs, and colchicine, over the same dose range at which these drugs inhibit other cytotoxic cells. We have no evidence that NK cell "specificities" are clonally distributed. Two sets of evidence are presented which suggest that the same NK cell population is responsible for lysing a variety of tumor target cells. Preliminary evidence suggests that modulation of NK cell levels *in vivo* is correlated with resistance to challenge with a syngeneic tumor, inferring that NK cells may play a salient role in host defenses against neoplasia. (Am J Pathol 93:459–468, 1978)

THE CONCEPT that the immune system is the principal regulatory mechanism for the control of neoplastic growth in mammals was first developed by Ehrlich and was later expanded by Thomas, Burnett, and others. Such notions were crystallized in the hypothesis of immune surveillance, which proposed that cells of the immune system "surveyed" the body for the development of malignant cells arising by somatic mutation, and, on "recognizing" such cells, destroyed them.[1,2] With the demonstration that certain lymphocyte subpopulations were capable of lysing tumor cells *in vitro*,[3,4] attention focused on cytotoxic lymphocytes as the effector arm of immune surveillance.[2]

The immune surveillance hypothesis has, however, recently come under attack from a number of quarters.[5-7] Broadly speaking, the criticism has fallen into three major categories: a) A failure to demonstrate tumor-specific transplantation antigens on an array of tumors, particularly those arising spontaneously.[8] This is seen by some as a forceful argument, since it is widely thought that such antigens represent the means by which tumor

From the Department of Medicine of the Johns Hopkins University School of Medicine and the O'Neill Memorial Research Laboratories of the Good Samaritan Hospital, Baltimore, Maryland.

Presented at the Sixty-second Annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 10, 1978.

Supported by Grants CA 22979 (to Dr. Henney) and CA 19483 (to Dr. Tracey) from the National Cancer Institute and by Grant AI 10280 (to Dr. Henney) from the National Institute of Allergy and Infectious Disease.

Address reprint requests to Dr. C. S. Henney, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

0002-9440/78/0119-0459/$01.00

cells can be recognized by the immune system. b) The observation that "nude" mice, which constitutionally lack a thymus and are thus incapable of generating cytotoxic T lymphocytes, are no more susceptible to neoplasms of either viral or chemical origin than are their normal littermates.[9,10] c) The finding that depression of the immune system is frequently not associated with an increased incidence of neoplasia.[10]

Just as these and other arguments are being refined and contended,[5-7] an observation has been made which potentially alters not only our appreciation of the immune system but also how it might function with respect to controlling neoplastic growth. The observation itself was rather simple: lymphoid cell populations from normal animals (mice, rats, and humans) were shown to be capable of lysing tumor cells, but not normal cells, in short-term *in vitro* assays involving ^{51}Cr release.[11,12] Considerable attention has been paid to a characterization of the cells responsible for this activity and to a preliminary exploration of their significance *in vivo*.

The lytic activity of normal lymphocyte populations, ie, those derived from non-tumor-bearing hosts, has been attributed to a class of cells termed "natural killer" (NK) cells. Such cells have best been characterized in the mouse (Table 1). The cells lack clearly demonstrable surface markers of mature mouse lymphocytes, but they are apparently confined to lymphoid tissue and are derived from a precursor in bone marrow.[13] Little is yet known of the mode of cytotoxic action of NK cells, although cytolysis can be modulated pharmacologically in a similar manner to that observed with other cytotoxic cell populations (Table 2).

The most startling aspect of the cytotoxic activity of NK cells in all species in which they have been defined is their apparent ability to discriminate between normal and "malignant" cell types. The former are

Table 1—Mouse Natural Killer (NK) Cells

Cell surface	Lacks Ig; Thy-1; Ia, Fc receptors; C3 receptors; Ly 1,2,3 May possess "NK antigen"
Cell characteristics	Nonadherent to glass and plastic; nonphagocytic; labile at 37 C
Specificity of lysis	Lymphomas especially sensitive; broad range of syngeneic, allogeneic, and xenogeneic tumor cells; cell lines sensitive; weak reactivity vs normal cells
Strain distribution	High in nudes, CBA, B6C3F_1, B6D2F_1, and C57BL/6; low in A, BALB/c Levels of reactivity polygenically controlled but partially linked to genes in the H-2 complex
Organ distribution	High in spleen, blood, lymph node; moderate in peritoneal exudate and bone marrow; absent in thymus
Effects of age	Absent at birth; peak levels 5 to 8 weeks; low after 12 weeks in most strains
Enhancement of activity	Caused by BCG, *Corynebacterium parvum*, and a variety of murine viruses, especially lymphocytic choriomeningitis virus

Table 2—Comparison of Drug Effects on NK-, K-, and T-Cell-Mediated Cytolysis

Drug	Dose	% Inhibition of specific cytolysis by: NK	T	K
Cytochalasin A	5 μg	100	98	
	1 μg	28	37	
Cytochalasin B	5 μg	94	97	85
	1 μg	12	23	19
PGE_1	10^{-5} M	72	62	68
$PGF_1\alpha$	10^{-5} M	2	0	3
Colchicine	10^{-4} M	72	68	80
	10^{-5} M	15	12	25

NK cells were obtained from peritoneal exudates of C57BL/6 mice 4 days after administration of 10^8 viable BCG organisms.[14] Alloimmune T cells were obtained from the spleens of C57BL/6 animals 10 days after immunization with 3×10^7 P815 cells.[15] Human peripheral blood lymphocytes were used as a source of K cells.[16] NK- and T-cell-mediated cytotoxicity were assessed using P815 cells; K-cell-mediated lysis was assessed using human Chang cell targets coated with a rabbit anti-Chang-cell serum.[16] In all cases, a 4-hour ^{51}Cr-release assay was performed in the presence or absence of the drug named and percent inhibition caused by drug was determined.

largely insusceptible to lysis in the presence of NK cells; the latter are susceptible.[17] Even among susceptible cells, however, it is clear that there is a hierarchy of susceptibility: lymphomas are consistently lysed to a large extent; carcinomas are frequently less susceptible. Interestingly, within a given species, this hierarchy of susceptibility is maintained regardless of the strain source of NK effector cells. In other words, there is no obvious requirement for a shared histocompatibility between effector and target cell for cytotoxic expression by NK cells. In this respect NK cells differ from many cytotoxic T cells.[18]

The description of NK cells is almost certain to revive interest in the generality of the thesis of "immune surveillance," for even Ehrlich in his most inventive mood could not have designed a cell better suited to survey against somatic mutations than one which can selectively lyse malignant cells regardless of their origin. Whether NK cells have anything to do with the immune system as currently defined or whether they represent a primordial cell type conserved because of obvious selective advantages awaits ontogenic and phylogenetic studies.

We have been particularly interested in asking whether NK cells are clonally distributed, as are immunocompetent lymphocytes and whether the heightened NK reactivity we have previously described following BCG infection [14,19] is due to polyclonal activation. One way we have chosen to address this issue is to investigate whether NK cells can be absorbed onto monolayers of susceptible cells and, if so, whether such associations remove NK reactivity against other susceptible target cells.

One experiment of this type is shown in Table 3. As can be seen, murine NK cell reactivity against L5178 lymphoma cells was selectively removed by incubation of effector cells on an L5178 cell monolayer but to a much lesser degree on a monolayer of normal DBA/2 spleen cells which are insusceptible to NK-mediated lysis. Concomitant with the removal of NK reactivity against L5178 cells, adsorption on the L5178 cell monolayer also removed NK reactivity against another susceptible cell line, ie, human Chang cells. In other studies (not shown) a reciprocal experiment was performed. NK cell reactivity toward both L5178 and Chang cells was totally removed by adsorption (at 37 C for 40 minutes) on Chang cell monolayers. The results of these experiments are incompatible with the concept that distinct subpopulations of NK cells lyse different target cells. Rather, they suggest that target cell susceptibility to NK action may be determined by a shared antigenic (?) specificity.

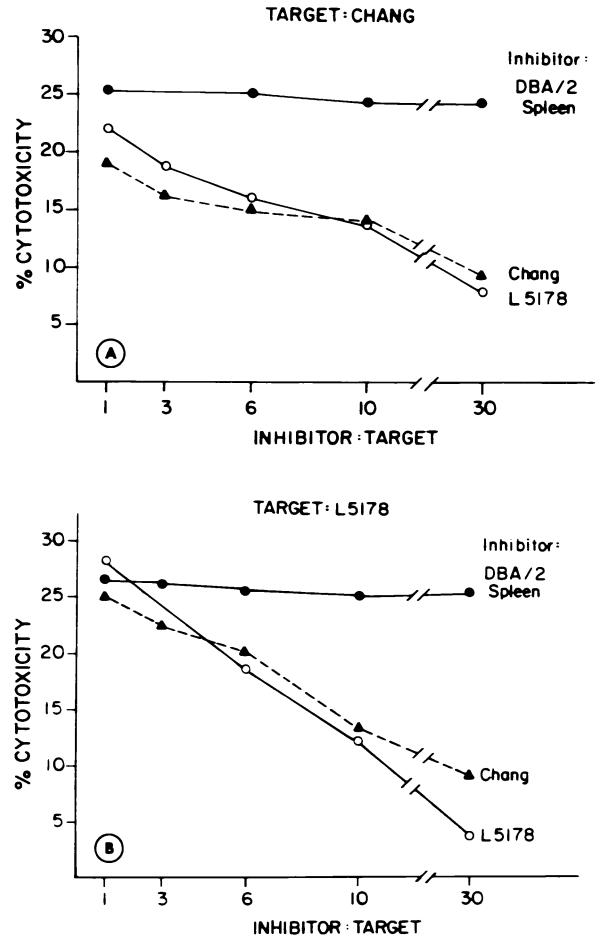
These conclusions were further supported by "cold" target inhibition studies, in which a variety of susceptible and insusceptible target cells were used to inhibit the NK-cell–mediated lysis of a susceptible cell line. Typical results (Text-figures 1A and B) illustrate an unequivocal finding: susceptible cell lines are capable of inhibiting the lysis of others; cells insusceptible to lysis are not inhibitory. Thus, the lysis of human Chang cells by NK cells from C57BL/6 mice was inhibited as well by L5178 cells as it was by homologous Chang cells (Text-figure 1A). Similarly, Chang cells inhibited the lysis of L5178 cells (Text-figure 1B). In neither experiment did normal spleen cells interfere with lysis (Text-figure 1).

These experiments provide no evidence for clonal distribution of NK

Table 3—Depletion of NK Cell Reactivity on Susceptible and Insusceptible Cell Monolayers

NK cell fractionation	Relative lytic activity toward: L5178	Chang	Normal DBA/2 spleen
Unfractionated	320	160	<10
Cells nonadherent to L5178 monolayers	32	28	<10
Cells nonadherent to DBA/2 spleen monolayer	110	72	<10

Peritoneal exudate cells were obtained from C57BL/6 mice 4 days after they had received 10^8 viable BCG organisms intraperitoneally. The activity of these cells toward ^{51}Cr-labeled target cells in a 4-hour assay was determined; the cells fractionated (for 40 minutes at 37 C) on poly-L-lysine induced monolayers of L5178 lymphoma cells or of DBA/2 spleen cells, and the reactivity of the nonadherent cells was re-assessed. The relative lytic activity is expressed in the form of the lytic units obtained by multiplying the cytolytic activity by the percent cell recovery following fractionation.



TEXT-FIGURE 1—Peritoneal exudates obtained from C57BL 6 mice 4 days after 10^6 viable BCG organisms were used intraperitoneally as a source of NK cells. The NK-mediated lysis of ^{51}Cr Chang cells (A) or ^{51}Cr-L5178 lymphoma cells (B) was followed in a 4-hour assay at 37 C in the presence of differing numbers of unlabeled Chang cells, L5178, or DBA/2 spleen cells. The percent cytotoxicity observed in the absence of inhibitory cells was 25% using Chang cell targets (A) and 28% using L5178 cells (B).

reactivities; rather, they suggest that the wide range of target cells which are killed by NK cells reflects the display of a common membrane "marker." There has been a suggestion that this marker might be related to C-type virus particle display,[17] but a recent study by Becker and Klein failed to establish such a relationship.[20] Although they may not throw light on the specificity of the NK-mediated lytic process, recent unpublished observations from this laboratory suggest that individual clones from the same parent tumor show a spectrum of susceptibilities to NK-mediated lysis, implying that it may be feasible to clone susceptible and insusceptible cell variants from the same tumor. Should this prove possible, the search for the molecular basis of NK susceptibility should be considerably aided.

While *in vitro* studies of NK cells are currently focused on the nature of the cell's recognition unit and its homologous "antigen" on the target cell, there have been several recent attempts to implicate NK cells in host defenses against neoplastic growth. Our own studies to evaluate the role of NK cells in tumor rejection have been based on our previous observations that NK cell reactivities in the peritoneal cavity can be markedly elevated by the instillation of viable BCG organisms.[14,19] BCG induces at least two distinct effector cells which are tumoricidal *in vitro*: a cytotoxic NK cell and a growth-inhibitory adherent cell.[21] Growth inhibition of tumor cells by BCG-induced macrophages [22] or nonphagocytic adherent cells [21] has been observed by other investigators using 48-hour *in vitro* assays based on the uptake of radiolabeled thymidine analogs by tumor cells.[21,22] NK-mediated reactivities are assessed by measurement of short-term (4-hour) ^{51}Cr release. Using these assay systems we have performed experiments to evaluate the relative potency of these different effector cells *in vivo*. To obtain a distinction between NK and growth inhibitory cells *in vivo*, we utilized the observation that the time courses of appearance of these cells after BCG inoculation are different. NK acitivity peaks 4 to 5 days after BCG inoculation [14]; in contrast, growth-inhibitory activity does not peak until 10 to 25 days.[22] We took advantage of this difference in the time courses of the two effector activities to construct the following experiments. Groups of C57BL/6 mice were inoculated intraperitoneally with BCG or thioglycollate medium 4 or 10 days prior to an inoculation with EL4 cells intraperitoneally. Growth of the EL4 tumor was assessed as the mean survival time of the tumor-bearing mice. As shown in Table 4, the only group which was significantly protected was the group which received BCG 4 days before tumor. Similarly, Table 5

Table 4—BCG-Induced Suppression of EL-4 Tumor Cell Growth

Treatment protocol*	% Cytotoxicity by PEC at day 0†	Mean survival time (days)	Significance (Student *t* test)
None (tumor alone)	−2.0	13.0 ± 2.4	$P < 0.01$
BCG Day −10	3.1	15.4 ± 0.9	
BCG Day −4	12.0	21.0 ± 5.0	
BCG Day 0	−1.9	17.9 ± 5.5	
THIO Day −10	−2.5	11.0 ± 0.0	
THIO Day −4	0.2	13.0 ± 1.7	
THIO Day 0	−3.3	9.1 ± 0.8	

* Groups of 4 to 8 C57BL/6 mice were inoculated intraperitoneally with 10^6 viable BCG or 2 ml 3% thioglycollate medium on Days −10, −4, or 0. All mice were inoculated with 5×10^4 EL-4 ascites cells intraperitoneally on Day 0.

† Cytotoxicity of ^{51}Cr-El-4 cells in 4 hours at an effector:target cell ratio of 50:1.

Table 5—BCG-Induced Suppression of L-5178 Tumor Growth

Treatment of tumor recipients*	% Cytotoxicity by PEC on Day 0†	% Growth inhibition by PEC on Day 0‡	Survivors	Mean survival time (days)
None	0.1	−75.3	0/8	32.8
BCG Day −5	37.6	92.7	4/8	56.3
BCG Day −20	11.2	96.3	0/8	35.1

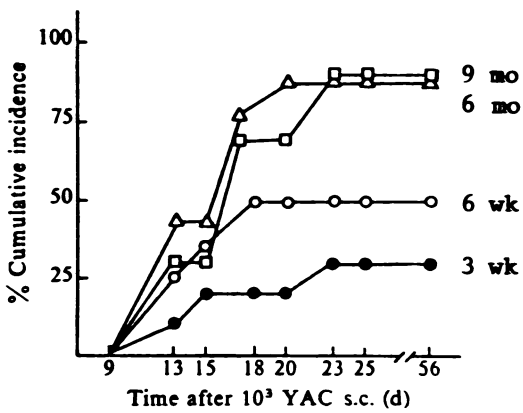
* B6D2F$_1$ mice were inoculated intraperitoneally with 10^6 viable BCG on Day −20 or −5. All mice were inoculated with 10^4 L-5178 ascites cells intraperitoneally on Day 0.

† Cytotoxicity of ^{51}Cr-L-5178 cells in 4 hours at an effector:target cell ratio of 33:1.

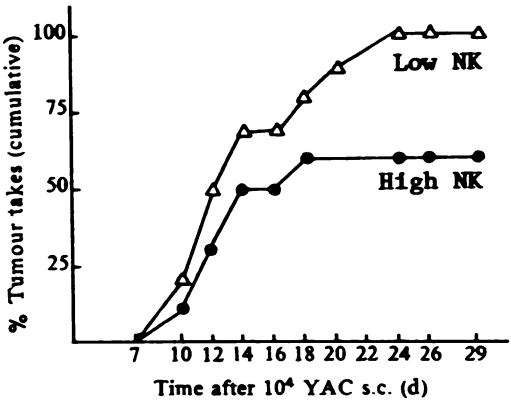
‡ Growth inhibition of L-5178 cells in a 42-hour ^{125}IUdR uptake assay at an effector:target ratio of 33:1.

shows that protection against the growth of L5178 tumor in B6D2F$_1$ mice was afforded by injection of BCG 5 days, but not 20 days, before tumor inoculation. It is striking that the 20-day BCG–PEC show 96% growth inhibition of these tumor cells *in vitro* but do not appear to be active *in vivo*. Results of these BCG prophylaxis experiments indicate that NK cells are more efficient than growth-inhibitory adherent cells *in vivo* in retarding tumor growth.

In other attempts to evaluate the tumoricidal activity of NK cells *in vivo*, Haller et al [23] made use of the age dependence of NK cell reactivity in mice.[24] (A/Sn×CBA/H) F$_1$ mice of varying ages were inoculated with a syngeneic tumor (YAC) and the incidence of tumor "takes" was measured at frequent intervals. The data derived (Text-figure 2) clearly demonstrated that young mice (which displayed high NK reactivity toward YAC cells *in vitro*) had a lower tumor incidence than did older mice in which NK cell reactivity had diminished. An even more persuasive correlation



TEXT-FIGURE 2—(A Sn × CBA/H) F$_1$ mice of differing ages were inoculated with 10^3 YAC cells subcutaneously. The incidence of animals bearing tumors was assessed at frequent intervals. (Data are adapted from Haller et al [23] with permission from *Nature*.)



TEXT-FIGURE 3—(A/Sn × C57BL/6) F_1 mice were irradiated (750 rad) and reconstituted with bone marrow from either (A Sn × C57BL/6) F_1 mice (*High NK*) or from (A/Sn × A.BY) F_1 mice (*Low NK*). Both groups of mice were given 10^4 YAC cells subcutaneously. The incidence of tumor "takes" was measured at frequent intervals. (Data are adapted from Haller et al [23] with permission from *Nature*.)

between NK cell reactivity and tumor resistance was provided by the same investigators using x-irradiated and bone-marrow–reconstituted mice as recipients of syngeneic tumor. (A/Sn × C57BL/6)F_1 mice were subjected to 750 rad and were then reconstituted with histocompatible bone marrow derived from mice which were either genetically "high" or "low" exhibitors of NK cell reactivity. Such manipulations produced mice which were identical with respect to histocompatibility but which showed distinctive variation in NK reactivity. All adult animals were given syngeneic (YAC) lymphoma cells; tumor incidence was then measured (Text-figure 3). Mice with high NK reactivity showed a significantly lower incidence of tumors than did animals reconstituted to display low NK reactivity.[23]

These sets of studies represent an initial, but already persuasive, body of data which implicates NK cells as a potentially important arm of host defense against neoplasia. Circumstantial evidence suggests that the immunotherapeutic efficacy of BCG in treating human malignancies may, at least in part, be attributable to the increased NK reactivity stimulated by this microorganism. Given that a wide variety of bacterial and viral products have been shown to stimulate NK reactivity (perhaps, as recently suggested,[25] via interferon production), we may soon see the birth of a new dictum in cancer immunotherapy: "stimulate the NK cells."

References

1. Thomas L: Discussion. Cellular and Humoral Aspects of the Hypersensitive States. Edited by HS Lawrence. New York, Hoeber-Harper, 1959, p 529
2. Burnett FM: The concept of immunological surveillance. Prog Exp Tumor Res 13:1–27, 1970
3. Govaerts A: Cellular antibodies in kidney homotransplantation. J Immunol 85:516–522, 1960
4. Cerottini JC, Nordin AA, Brunner KT: Specific *in vitro* cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. Nature (Lond) 228:1308–1309, 1970

5. Melief CJM, Schwartz RS: Immunocompetence and malignancy. Cancer, a Comprehensive Treatise, Vol 1. Edited by FF Becker. New York, Plenum Publishing Corp., 1975, pp 121–159
6. Stutman O: Immunodepression and malignancy. Adv Cancer Res 22:261–422, 1975
7. Prehn RT: Immunological surveillance: Pro and con. Clinical Immunobiology, Vol 2. Edited by FH Bach, RA Good. New York, Academic Press, Inc., 1974, pp 191–203
8. Prehn RT: The relationship of immunology to carcinogenesis. Ann NY Acad Sci 164:449–457, 1969
9. Stutman O: Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science 183:534–536, 1974
10. Stutman O: Immunodeficiency and cancer. Mechanisms of Tumor Immunity. Edited by I Green, S Cohen, R McCluskey. New York, John Wiley & Sons, Inc., 1977, pp 27–53
11. Herberman RB, Nunn ME, Lavrin DH: Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer 16:216–229, 1975
12. Kiessling R, Klein E, Wigzell H: "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 5:112–117, 1975
13. Haller O, Wigzell H: Suppression of natural killer cell activity with radioactive strontium: Effector cells are marrow dependent. J Immunol 118:1503–1506, 1977
14. Tracey DE, Wolfe SA, Durdik JM, Henney CS: BCG-induced murine effector cells. I. Cytolytic activity in peritoneal exudates: An early response to BCG. J Immunol 119:1145–1151, 1977
15. Henney CS: Quantitation of the cell-mediated immune response. I. The number of cytolytically active mouse lymphoid cells induced by immunization with allogeneic cells. J Immunol 107:1558–1566, 1971
16. Ziegler HK, Geyer C, Henney CS: Studies on the cytolytic activity of human lymphocytes. III. An analysis of requirements for K cell-target interaction. J Immunol 119:1821–1829, 1977
17. Herberman RB, Holden HT: Natural cell-mediated immunity. Adv Cancer Res 1978 (In press)
18. Zinkernagel RM, Doherty PC: Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis virus within a syngeneic or semiallogeneic system. Nature (Lond) 248:701–702, 1974
19. Wolfe SA, Tracey DE, Henney CS: BCG-induced murine effector cells. II. Characterization of natural killer cells in peritoneal exudates. J Immunol 119:1152–1158, 1977
20. Becker S, Klein E: Decreased "natural killer" effect in tumor-bearing mice and its relation to the immunity against oncorna virus-determined cell surface antigens. Eur J Immunol 6:892–898, 1976
21. Nathan CF, Hill VM, Terry WD: Isolation of a subpopulation of adherent peritoneal cells with anti-tumour activity. Nature 260:146–148, 1976
22. Hibbs JB, Lambert LH, Remington JS: Possible role of macrophage mediated nonspecific cytotoxicity in tumour resistance. Nature [New Biol] 235:48–50, 1972
23. Haller O, Hansson M, Kiessling R, Wigzell H: Role of non-conventional natural killer cells in resistance against syngeneic tumor cells *in vivo*. Nature 270:609–611, 1977
24. Kiessling R, Klein E, Pross H, Wigzell H: "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol 5:117–121, 1975
25. Wigzell H, Herberman R: Personal communications

[*End of Article*]