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Age-associated impairment of murine natural killer activity

(aging/parasitic infection/natural resistance)

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ABSTRACT **Natural cell-mediated cytotoxicity may be critical in the resistance displayed by animals and humans to tumors and various pathogenic microorganisms. Because the frequency of tumors and infections increases markedly in aging populations, we have compared the natural killer (NK) competence of lymphoid tissues (spleen, bone marrow) and of peritoneal cells of young adult and aged mice. Spontaneous NK activity was much lower, and the rate of target cell lysis was much less, in aged mice. The level of NK activity was only modestly increased in old, compared to young, mice when they were exposed to *Trypanosoma musculi*, an organism that provides strong stimulation of NK activity. Restricted NK activity of aged mice was not attributable to suppressor cells. The NK effector cells in old mice were characterized as being nonadherent to plastic, completely susceptible to lysis by complement plus an antiserum against specificity NK 1.2, and only slightly affected by treatment with antiserum against specificity Thy-1.2. Two indirect methods were employed to assess the relative frequency of splenic NK cells at the time of maximal stimulation by *T. musculi*: a cytotoxicity assay with antiserum against NK 1.2 and a binding assay involving monolayers of YAC-1 tumor target cells. Similar results were obtained in both assays, indicating that at maximal stimulation about 10% of the total spleen cells of both young and old mice were NK cells. We conclude (cautiously) that the functional efficiency of aged NK cells is impaired and that this defect may account, in part, for reduced ability of aged individuals to resist certain types of cancer and certain pathogenic microorganisms.**

Many biological and medical scientists believe that the declining competence of the immune system is responsible, in part, for the progressive rise in the frequency of neoplastic and infectious diseases associated with biological aging. The veracity of this postulate could be better judged if more were known about the immunological processes involved in responses to, and reactions with, tumor cells and pathogenic organisms. One feature of the immune system that has received considerable attention in recent years is that of spontaneous, or natural, cell-mediated cytotoxicity, associated especially with the subset of lymphocytes termed "natural killer" (NK) (1, 2). A variety of tumors (3, 4) and several pathogens (5–7) have been found to be susceptible to NK cells. The most clear-cut example of the susceptibility of a pathogen to NK cells is that of the human pathogen *Trypanosoma cruzi* (7). In addition, a number of other protozoan parasites have been found to alter markedly the NK activity of lymphoid tissues of their hosts (8–12).

At present, information about the status of NK cells and their functional capabilities in aging animals and humans is meager. In most strains of mice, NK activity in lymphoid tissues is highest in mice that are 1–3 months of age and declines considerably thereafter (13–15). Little is known about NK cells and activity in aged mice. Cells described as large granular lymphocytes effect NK activity in mice (16) as well as in rats (17) and humans (18). There is evidence that the frequency of large granular lymphocytes in the spleens of aging mice declines in concert with decline in NK activity (16). Quite recently it has been reported (19) that splenic NK activity is very low in aged mice maintained on either conventional or restricted (reduced caloric intake) diets; however, upon stimulation with an interferon-inducing agent, poly(inosinic acid)·poly(cytidylic acid) [poly(I·C)], aged mice maintained on the restricted diet developed substantially higher levels of NK activity than those fed the conventional diet. It was suggested that this competence to generate NK activity might, in part, account for the significantly reduced incidence of tumors in rodents reared on restricted diets (20). A somewhat different result was reported in the case of natural cell-mediated cytotoxicity (NC), an activity associated with a closely related but probably distinct subset of lymphoid cells (21). In this case, little or no decline in splenic NC activity was detected in several strains of aged mice.

We have analyzed the lymphoid tissues of several strains of aging inbred mice with respect to both spontaneous and induced NK activity. For the latter purpose, the mouse-specific parasite *Trypanosoma musculi* was employed because, as we have shown (12), inoculation with this parasite increases markedly the NK activity of spleen, bone marrow, and peritoneal cells and blood. In addition, this investigation has been concerned with certain properties of the cells responsible for NK activity in aged mice and with an evaluation of the functional efficiency of these NK cells.

MATERIALS AND METHODS

Mice. Two inbred strains and a hybrid line of mice were used. Mice of the C3H/Anf Cum strain as well as the $BC3F_1$ hybrid (C57BL ♂ × C3H/Anf ♀) were purchased from Cumberland View Farms (Clinton, TN) at 5–6 weeks of age. Mice of the C57BL/6J strain were purchased from The Jackson Laboratory. Mice were maintained in plastic pans situated in laminar-flow animal racks. Water bottles and pans were replaced twice per week. The ages of the mice employed in the experiments were young, 3–5 months; old, 24–30 months.

At sacrifice, mice were anesthetized and killed by cervical dislocation. Cells were removed from the peritoneal cavity by washing it with warm culture medium. Bone marrow cells were dispersed by gentle aspiration with a glass pipette. The spleens were teased and the dispersed cells were washed by low-speed centrifugation. Cell viability was assessed by erythrosin B dye exclusion.

Trypanosomes. The origin, maintenance, and handling of *T. musculi* in our laboratory have been thoroughly described (22, 23). Mice received 5×10^5 viable trypanosomes at inoculation, and were sacrificed at appropriate times thereafter. Our use of *T. musculi* to stimulate NK activity is based on a previous in-

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Abbreviations: NK, natural killer; NC, natural cytotoxicity.

vestigation (12), from which it was learned that a parasite infection provides a powerful stimulus, at least as effective as injection of poly(I·C) or, in comparison to other reports, as effective as bacterial endotoxin, Newcastle disease virus (24), or carrageenan (25) vis-a-vis NK stimulation.

YAC-1 Tumor Cells. This is a line of cultured cells derived originally from a lymphoma induced in A/Sn mice by the Moloney leukemia virus. It is widely used as a target for NK cells. A sample of the YAC-1 cell line was graciously provided to us by Julie Djeu. In preparation for use as target cells, the tumor cells were harvested from cultures during exponential growth. They were labeled with ^{51}Cr by incubation in medium containing $Na^{51}CrO_4$ (New England Nuclear) then washed and counted by use of hemacytometers.

NK Assay Procedure. The procedure employed was patterned after the procedure in common use (e.g., see ref. 26). In brief, a constant number of ^{51}Cr-labeled tumor cells and graded numbers of test effector cells were mixed in the wells of microtiter plates (Flow Laboratories). The plates were placed in the CO_2 incubator and remained there until the cultures were terminated. Upon removal from the incubator, aliquots of the supernatants from each well were removed for measurement of released ^{51}Cr. Spontaneous release of isotope was estimated by incubating the tumor cells alone. Maximal isotope release was estimated by brief exposure of the labeled cells to 1% Triton X-100. The percent specific cytolysis was estimated by use of the following formula:

$$\% \text{ specific release} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100.$$

Antiserum. Mouse anti-Thy-1.2 serum was purchased from Bionetics (Charleston, SC) and used at the predetermined optimal dilution. Mouse anti-NK 1.2 serum was a gift from Robert Burton, who also supplied low-toxicity rabbit serum as a complement source; we are grateful for his generosity. The preparation of this antiserum and appropriate applications of the serum have been described (27).

Cell Separation Procedures. Our version of the procedure for separating murine cells according to their affinity for plastic surfaces has been described (28). The preparation of gradients of Percoll was based on literature supplied by the manufacturer (Pharmacia). In brief, stock Percoll solution was diluted with physiological salt solutions to obtain the desired concentrations at isosmotic condition. Discontinuous gradients were prepared from Percoll solutions that varied (in increments of 10%) from 80% (bottom layer) to 40% (top layer). Murine cell suspensions were layered on top of tubes of gradient and centrifuged (1,065 × g for 20 min). The layers of differing concentration with the included cells were recovered, the Percoll was diluted with culture medium, and the cells were recovered by centrifugation.

Adsorption of NK Cells on YAC-1 Monolayers. This procedure was based on a similar procedure described by other investigators (29, 30). An alternative procedure (31) for enumeration of NK cells was not employed because it provided highly variable results when applied to NK cells obtained from *T. musculi*-infected mice. Polystyrene culture plates were coated with poly(L-lysine) (Sigma), then washed three times with phosphate-buffered saline (no Ca^{2+} or Mg^{2+}). YAC-1 cells were collected during exponential growth and washed in tissue culture medium (without serum). Each polylysine-coated dish was provided with 1×10^7 YAC-1 cells and the dishes were incubated for 30 min at room temperature. The medium was decanted and immediately a solution of cold 0.2% formaldehyde (in phosphate-buffered saline) was added to the dishes. After standing 1 hr at 4°C the fixed cells were washed five times with phosphate-buffered saline and left standing in this buffer for 40 min. After two additional washes, the dishes were ready for use. Two milliliters of a suspension containing 1.2×10^7 spleen cells in medium RPMI 1640 (fortified with 10% fetal bovine serum) was gently added to each dish. The dishes were placed in a tissue culture incubator at 37°C for 1 hr. Upon removal from the incubator the dishes were agitated, the unbound cells were decanted, the dishes were washed, and the washings were combined with the suspension of unbound cells. Finally, the unbound cells recovered from each dish were counted and the proportion of bound cells was calculated.

RESULTS

The levels of spontaneous, splenic NK activity toward YAC-1 target cells of young and old mice are depicted in Fig. 1, together with the changes in NK activity accompanying infections with *T. musculi*. This parasite is at least as effective in raising the level of NK activity as any stimulant reported to date (12). Two strains of inbred mice are depicted: C57BL/6 [a strain that is relatively resistant to *T. musculi* infection, (32)] and C3H (a susceptible strain). Young and old mice of the $BC3F_1$ hybrid line were studied also and the results (not shown) agreed well with those depicted in Fig. 1. The rise to a peak on day 3 and the subsequent rapid decline in NK activity in young-adult mice confirms previous results (12). It is apparent that the changes in splenic NK activity in the infected old mice were much less pronounced. From a spontaneous level that was significantly below that in young-adult mice, the NK activity did increase to some extent in old mice, but far from that displayed in young mice. There was little, if any, difference in NK activity of the spleen in infected mice of the two strains, either young or old. In a separate experiment involving a small group of six aged mice, we found that injection of poly(I·C) into infected aged mice was unable to increase splenic NK activity beyond the level found in aged mice that were only infected with *T. musculi* (data not shown).

To gain additional insight concerning the defective splenic NK activity of aged mice, the rates of target YAC-1 cell lysis

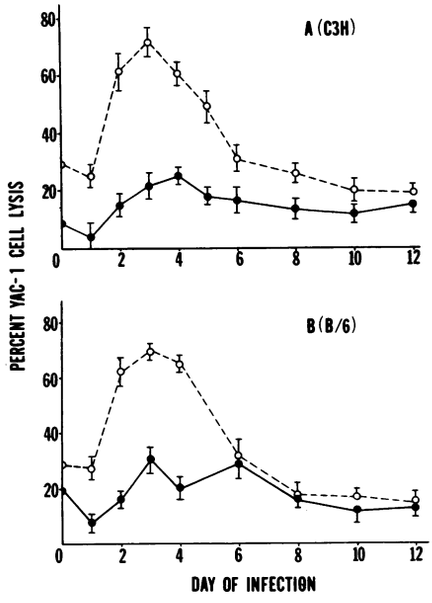

FIG. 1. NK activity of spleen cells taken at intervals during the course of *T. musculi* infections of young (○) or old (●) mice of strain C3H (*A*) or C57BL/6 (*B*). Results are from four separate experiments involving 12–16 mice per point; vertical bars are 1 SEM. Ratio of effector spleen cells to target YAC-1 cells was 200:1.

were measured with effector cells from normal or infected, young or old, donor mice. The data, presented in Fig. 2, disclose that the rates of target cell lysis varied considerably. Per unit number of spleen cells, both the rate of lysis and the magnitude of lysis were greatest in the case of young 3-day-infected donors; by both indices the activity of infected donor spleen cells was much greater than in the case of young normal donor cells. The defective spontaneous NK activity of normal old spleen cells was revealed in both the rate and magnitude of target cell lysis. The NK activity increased somewhat in the infected old mice; spleen cells from the latter donors were equivalent, in both rate and magnitude of target cell lysis, to spleen cells from normal young mice. The kinetics of target cell killing are graphed individually for three old normal and three old infected mice in Fig. 2*B*. The reactions displayed are representative and show that there was not extreme variation in NK activity among the old C57BL/6 mice employed.

The age-related decline in NK competence of the spleen prompted two immediate questions: (*i*) are similar changes apparent in sites of NK cells other than spleen; and (*ii*) are NK cells of aged mice similar to those of young adult mice? To answer the first question we analyzed the NK activity of peritoneal cells (Table 1) and of bone marrow (results not shown). The data in Table 1 are the activities of peritoneal cells and spleen cells of young and old, normal and infected, mice. In addition, these preparations of cells were subjected to partitioning into adherent and nonadherent populations according to their affinity for binding to plastic surfaces (Petri dishes). The NK activity of the peritoneal compartment of cells rose and fell in infected mice in concert with the changes in splenic NK activity. In general, the NK activity was associated with plastic-nonadherent cells. In the case of peritoneal cells collected from young infected donors, a portion of the NK activity on both day 3 and day 12 of infection appeared to adhere to plastic.

Further analyses of the properties of NK cells from young and old, normal and infected, mice involved testing their susceptibility to lysis by specific antisera directed against surface antigens. An antiserum specific for the T-cell antigen Thy-1.2 was used along with an antiserum specific for the NK cell antigen NK 1.2. The ability of these antisera to eliminate YAC-1

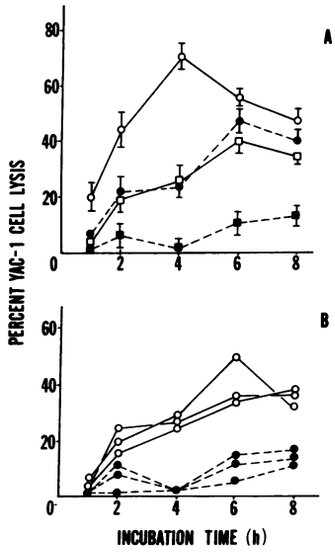

FIG. 2. Kinetics of YAC-1 target cell lysis by effector spleen cells. h, Hours. (*A*) Mean values of three experiments with effector cells from young infected donors (○), young normal donors (●), old infected donors (□), or old normal donors (■); vertical bars represent 1 SEM. (*B*) Individual rates of lysis by cells from three representative normal (●) or infected (○) old mice. Effector cell to target cell ratio, 200:1.

Table 1. Partitioning of NK activity with the plastic-nonadherent population

Age of mice*	Type of cells†	% YAC-1 cell lysis‡ by effector cells from		
			Infected mice	
		Normal mice	Day 3	Day 12
Young	Whole spleen	22.3 ± 5.4	62.5 ± 6.1	12.7 ± 5.1
Young	Nad spleen	28.9 ± 4.8	68.2 ± 5.8	18.1 ± 4.5
Old	Whole spleen	12.3 ± 4.6	31.9 ± 6.1	15.2 ± 5.9
Old	Nad spleen	14.1 ± 3.9	43.4 ± 4.9	17.9 ± 5.2
Young	Whole PT	8.4 ± 3.8	81.0 ± 7.8	45.4 ± 6.0
Young	Nad PT	7.8 ± 4.2	62.2 ± 6.5	34.3 ± 4.4
Old	Whole PT	3.0 ± 4.2	27.8 ± 5.1	18.4 ± 5.1
Old	Nad PT	4.6 ± 3.9	29.6 ± 4.8	16.1 ± 3.8

* Young mice, 3–4 months of age; old mice, 24–28 months of age.
† Nad, nonadherent; PT, peritoneal cells.
‡ Effector-to-target ratio, 200:1. Data are mean ± SEM.

target cell killing activity from the various spleen cell suspensions is reported in Table 2. These data are convincing, for they show that: (*i*) treatment with anti-NK serum eliminated virtually all effector cell activity from suspensions of normal young and old spleen cells and, similarly, from cell suspensions of young and old infected mice; and (*ii*) treatment with anti-Thy serum reduced NK activity only slightly, as expected (33, 34).

Having demonstrated that aged mice suffer from deficient NK competence and that the NK activity which remains is expressed by typical NK cells, we wondered whether or not the actual number of NK effector cells was substantially less in old than in young-adult mice. We have employed two procedures to obtain the approximate numbers of NK cells in the spleens of young and old infected mice: (*i*) adsorption of NK cells by YAC-1 monolayers, and (*ii*) cytotoxicity assays employing anti-NK serum and complement. The results obtained by application of the latter procedure are provided in Table 3. As a check on the precision of the procedure, cytotoxicity of anti-Thy serum was tested in parallel. Anti-NK serum was utilized in relatively high, as well as in dilute, antibody concentrations (see ref. 27). Inspection of the data in Table 3 discloses the following: (*i*) about 14% more cells of infected young donors than of normal young donors were killed by the relatively concentrated anti-NK serum (42–28%) and about 5% more were killed by the relatively dilute serum (20–15%); (*ii*) with respect to the spleen cells of old mice, about 12% more cells from infected mice, in comparison

Table 2. Effects of treating spleen cells with antiserum to NK 1.2 and Thy-1.2 on NK activity

Spleen cell donors*		Serum treatment	% YAC cells lysed†
Age	Condition		
Young	Normal	Control	26.3
Young	Normal	Anti-NK	4.4
Young	Normal	Anti-Thy	21.3
Old	Normal	Control	18.9
Old	Normal	Anti-NK	4.3
Old	Normal	Anti-Thy	16.2
Young	Infected	Control	61.6
Young	Infected	Anti-NK	12.6‡
Young	Infected	Anti-Thy	53.9
Old	Infected	Control	34.5
Old	Infected	Anti-NK	10.9‡
Old	Infected	Anti-Thy	31.4

* Young mice, 3–4 months of age; old mice, 24–28 months of age. Infected refers to 3-day infections with *T. musculi*.
† Effector-to-target ratio, 200:1.
‡ Residual activity eliminated by a second cycle of treatment with anti-NK serum plus complement.

Table 3. Killing of normal and infected mouse spleen cells with antisera against Thy-1.2 and NK 1.2

Condition of mice*	Antiserum		% spleen cells killed†	
	Specificity	Dilution	Young mice	Old mice
Normal	Thy	—	32	31
Infected	Thy	—	28	31
Normal	NK	1:30	28	38
Normal	NK	1:75	15	18
Infected	NK	1:30	42	50
Infected	NK	1:75	20	22
Normal	None‡	—	7	5
Infected	None‡	—	3	4

*Infected refers to 3-day infections with *T. musculi*.
†Effector-to-target ratio, 200:1. Young mice, 3–4 months of age; old mice, 24–28 months of age.
‡Control, normal rabbit serum; normal mouse serum control not significantly different.

to normal, were killed by the more concentrated antiserum (50–38%) and about 4% more were killed when the dilute antiserum was used (22–18%). Results quite similar to those in Table 3 have been obtained consistently in three separate experiments. From the results obtained with the more concentrated antiserum, it appears that some 12–14% more NK cells may be present in infected than in normal mice and that this number applies to both young and old mice. It should be stressed that the proportions of young and old spleen cells killed by anti-Thy or anti-NK serum were quite similar.

The second procedure that was applied to evaluating relative numbers of NK cells in young and old mice involved adsorption of NK cells to YAC-1 target cell monolayers. The results of four separate experiments are presented in Table 4. Results of the four experiments were similar. There was considerable nonspecific binding of spleen cells to the YAC-1 monolayers. However, without exception, more cells were bound when spleens from infected mice were tested than was the case with spleens from normal mice. About 16% more cells of infected young mice were bound than of normal young mice (Table 4, last column). Similarly, 12% more cells of infected old mice were bound than of normal old mice.

The results of the two approaches to evaluating the relative numbers of NK cells in young and old mice subjected to strong NK stimulation from the *T. musculi* infections are in agreement, at least, and suggest that young and old animals may have roughly the same potential for generating splenic NK cells. If this conclusion is correct, the possibility must be raised that the depressed NK activity in old mice is a reflection of increased activity of one or more suppressor populations. We have examined this possibility by testing NK activity of different subsets of spleen cells separated according to their density in discontinuous Percoll gradients.

Table 4. Binding of spleen cells from young and old, normal and infected, mice on YAC-1 cell monolayers

Spleen cell donors		% YAC-1-adherent				
Age	Condition	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Mean
Young	Normal	57	57	49	53	54 ± 3.3
Young	Infected	71	73	66	68	70 ± 2.3
Old	Normal	44	45	52	42	46 ± 3.8
Old	Infected	57	59	64	52	58 ± 4.3

Age and infected are as in previous tables. Means are presented ± SD.

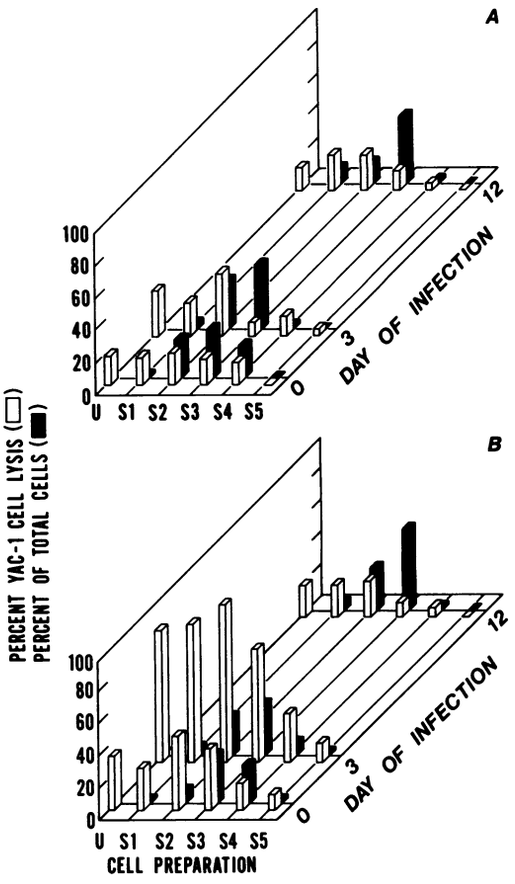

FIG. 3. NK activity (□) and total cell number (■) of preparations of spleen cells obtained from normal mice or mice on days 3 and 12 of *T. musculi* infection. Cell preparations as follows: U, whole unseparated spleen cells; S1–S5, fractions recovered in layers of discontinuous Percoll gradients ranging from 40% (S1) through 80% (S5). (*A*) Old mice (24–28 months); (*B*) young mice (3–5 months). Effector-to-target cell ratios were 200:1 in all cases.

Spleen cells from young and old, normal or infected, mice were introduced into tubes containing layers of graded concentrations of Percoll. After centrifugation the cells in each layer were recovered, washed, and assayed for NK activity. The results are depicted in Fig. 3. Identical experiments were performed with bone marrow rather than spleen cells (data not shown). It is apparent that NK activity was displayed by cells of different densities. It is apparent also that the cells present in some fractions (e.g., S2) displayed considerably more NK activity than was the case of the initial spleen cell suspension. However, there was no evidence that: (*i*) spleen cell preparations from infected mice contained inordinate numbers of suppressor cells, or (*ii*) the spleen of either normal or infected aged mice contained an abnormal proportion of suppressor cells.

DISCUSSION

Our investigation has revealed a substantial decline in aged mice in the competence of NK cells to effect lysis of YAC-1 target cells. Furthermore, the response of NK cells in old mice to stimulation with *T. musculi* (12) was considerably less than in similarly infected young mice. This deficient response in aged mice was not attributable to any disproportionate influence of suppressor cells. Rather, the evidence indicated that the rate of target cell destruction by NK cells of old mice was appreciably slower than by NK cells of young mice. Two indirect methods were employed to assess the frequency of NK cells in

the spleens of trypanosome-infected young and old mice (27, 29). Owing to the high background involved in both of these methods they could not be employed to assess accurately the number of NK cells in normal mouse tissues (nor are they normally used for that purpose). However, we have obtained reproducible results in estimating the NK cell frequency in trypanosome-infected mice. The results suggest that in the spleens of infected mice the frequency of NK cells at the time of peak response may be around 10% (data range, 12–16%). But the major point to extract from these results is that young and old mice have about the same potential for generating NK cells.

If this conclusion is reliable, it follows that NK cells of old mice are defective functionally. That would not be surprising, because considerable evidence exists for functional defects in various types of lymphohematopoietic cells of aged animals, including T lymphocytes (35, 36), B lymphocytes (37, 38), and hematopoietic stem cells (39).

The special importance of an age-associated deficiency in the competence of NK cells relates to the postulated role of these cells in resistance to neoplasias (40, 41) and pathogens (5–12). It is common knowledge that the incidence of many cancers and of infectious diseases (such as pneumonia and influenza) increases steadily in aging populations. There have been comprehensive studies of the rising frequency of certain cancers (e.g., lymphomas) in aging mice of long-lived strains (e.g., ref. 42). Laboratory investigations of age-associated susceptibility to pathogens have been less common. However, in the case of the murine pathogen *T. musculi*, we have completed an investigation which revealed that aged mice of several inbred strains suffer much more severe infections than do young-adult mice (43). It is reasonable, therefore, to envision declining NK competence as being particularly important in the pathogenesis of some of the diseases associated with aging. We will discuss elsewhere the complex question of whether or not NK cells are involved in combating *T. musculi* infections.

The results presented here agree generally with those contained in a recent report concerned with the influence of dietary restriction on NK activity in aged mice (19). In that report it was shown that aged mice reared on a restricted diet responded vigorously to NK stimulation by poly(I·C). In contrast to the evidence presented here and in ref. 19 that NK activity is quite low in lymphoid tissues of aged mice, it appears that little decline in NK activity occurs in peripheral blood of aging mice (44). It is possible that peripheral blood does not accurately reflect the status of the NK cell population in aged individuals.

As mentioned in the Introduction, the related but clearly distinct natural cytotoxicity for certain tumors (e.g., chemically induced solid tumors) does not appear to decline with age (21). It will be informative to determine whether or not there is a clear difference in the age-related incidence of tumors that are attacked by NK effector cells and those that are susceptible to NC effector cells. It is also important to determine, with precision, the numbers and functional efficiencies of NK cells from young and old animals and to investigate methods for retarding, as well as reversing, the decline of NK competence in aged individuals.

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