Lack of Correlation between Natural Killer Activity and Tumor Growth Control in Nude Mice with Different Immune Defects

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ABSTRACT

To elucidate the in vivo role of natural killer (NK) cells, the growth of several murine and human tumors was studied in four variants of athymic, nude mice with different levels of NK activity.

Beige-nude mice, homozygous for both the beige and the nude genes, had very low levels of NK activity, and their response to the B-cell mitogen, bacterial lipopolysaccharide, was lower than that of high-NK, adult NIH nude mice. Young and adult NIH nude mice had different NK levels and showed different responses in assays for K-cell, T-cell, and B-cell activity. The B-cell-defective NIH-II mice had slightly lower NK levels than adult NIH animals, but much lower response in the antibody-dependent cell-mediated cytotoxicity assay.

No correlation was found between host NK activity and the s.c. growth of various human (LOX, CEM, K562) and murine (YAC-1) tumor cells. Low NK activity was not associated with increased lung colony formation in a metastasis model using i.v.-injected human (LOX) and murine (B16F10) melanoma cells. No relationship was found between host NK activity and the rate of elimination of i.v.-injected 5-iodo-2'-deoxyuridine-labeled LOX, B16F10, and YAC-1 cells from lungs, liver, or spleen.

The results fail to support the view that NK cells exert significant direct effects on tumor cells in vivo.

INTRODUCTION

It is widely believed that NK cells have an important role in immune surveillance against tumors (12, 19, 36). The data supporting this theory have been obtained in mice with different levels of NK activity (23, 26), or in models where the animals were treated with drugs that are supposed to selectively affect NK function (11, 14, 15, 24). However, differences in host immune response other than in NK activity may have influenced the results. Therefore, definitive studies, proving the in vivo role of NK cells, are lacking (28), and there is a need for better models (33, 34).

Recently, we have reported on viable beige-nude mice with very low NK activity, compared to that of adult, regular NIH nude mice.3 Apart from the NK difference, the 2 types of nude mice differed immunologically only in a lower response to the B-cell mitogen, LPS, in the beige than in NIH animals. The beige-nudes, therefore, seemed to offer new possibilities for investigating the in vivo importance of NK cells.

In the present study, we have compared cellular immune response and tumor growth in beige-nude mice and in 3 other variants of athymic, nude mice with different immune characteristics. Surprisingly, no correlation between NK activity and tumor growth control was found.

MATERIALS AND METHODS

Mice

Four groups of immune-deficient, athymic mice were studied: (a) adult (5 to 7 weeks old) N:NIH(S) nude mice, which have high NK activity (18); (b) immunologically immature 2- to 3-week-old N:NIH(S) nude mice (14, 15, 18); (c) low-NK beige-nude mice, developed on C57BL/6N-beige and N:NIH(S)-nude background; and (d) B-cell-defective N:NIH(S)-II nude mice (1).

All animals were bred and kept in specific pathogen-free facilities until they had reached the desired age and body weight (adult mice, 5 to 7 weeks old), when they were transferred to conventional facilities for the tumor experiments. There, the mice were kept in disposable plastic cages with autoclaved filter tops, placed in laminar air-flow benches. The food and bedding were autoclaved before use, and the animals were given tap water ad libitum in sterilized bottles.

Breeding of Beige-Nude Mice

Five mating steps were needed to obtain beige-nude mice. (a) Female C57BL/6N-bg/bg +/+ mice were mated to male, nude N:NIH(S) +/+ nu/nu mice, resulting in bg/bg +/+ offspring, agouti in color. (b) The bg/ +/+ mice were crossed back to C57BL/6N-bg, yielding 50% homozygous bg/bg beige mice. Half of these were nu/+, and half were +/+; but these could not be distinguished by eye. (c) When such beige mice were mated to either nu/+ females or nu/nu males, the bg/bg +/+ parent could be identified, since they had at least one nude animal in their litter. (d) Female and male bg/bg +/+ mice were mated to each other, producing one-fourth beige-nude bg/bg bg/nu, two-fourths bg/bg bg/nu, and one-fourth bg/bg +/+ animals. (e) To identify the bg/bg +/+ beige-nude males were mated to bg/bg nonnude females. Those females that did not produce any nude offspring were bg/bg +/+ and therefore discarded, whereas the remaining bg/bg +/+ females were used to establish the breeding colony, together with the bg/bg bg/nu males. Since the progeny were either bg/bg bg/nu or bg/bg bg/nu, in equal proportions, no further testing was necessary.

Tumors

Two murine (YAC-1 and B16F10) and 3 human (K562, CEM, and LOX) tumors were used. The YAC-1 is a lymphoma, originally induced in A/Sn mice, and the cell line is commonly used as a sensitive target for testing NK cell activity in vitro (4). The B16F10 melanoma cell line, isolated by Fidler (7), was obtained from E. M. Jensen, Mason Research Institute of Experimental Therapy and Metabolism, Developmental Therapeutics Program, Division of Cancer Treatment, Bethesda, Maryland 20894.
Institute, Worcester, MA. The human, undifferentiated T-cell line, CEM, was a gift from Kevin Scanlon, Mt. Sinai Medical Center, New York, NY. The K562 line is a chronic myelogenic cell line, originally established by Lozzio and Lozzio (29). LOX is a human malignant melanoma, passaged as a xenograft tumor line in nude mice, and also established as a cell line in vitro.

The tumor cells were kept in continuous culture in RPMI 1640 medium (Flow Laboratories, Rockville, MD) with 5% fetal calf serum for at least 3 to 4 weeks, to ensure a high sensitivity to NK cells (2, 13), before being used in the experiments.

The tumors were shown to be free of Mycoplasma and the following pathogenic viruses: (a) K-virus; (b) reovirus type 3; (c) pneumonia virus of mice; (d) Thiel’s encephalitis virus; (e) Sendai virus; (f) minute virus of mice; (g) mouse adenovirus; (h) mouse hepatitis virus; (i) lymphocytic choriomeningitis virus; and (j) ectromelia virus (Microbiological Associates, Bethesda, MD).

Cellular Immune Response In Vitro

NK and ADCC Cytotoxicity Assays. In the NK cell assay, spleen cells from the different groups of mice were dispersed (0.1 ml) at different concentrations into the round-bottomed wells of a microtiter plate. 51Cr-labeled YAC-1 tumor cells (0.1 ml) were added into the appropriate wells and incubated at 37°C for 4 hr. The ADCC assay, regarded to reflect the activity of killer cells (34), was performed essentially as described (16), but with Chang cells as targets. The rabbit anti-Chang antisera was a gift from Julie Djeu, and was used in an optimal concentration of 1/10. The antibody-coated 51Cr-labeled Chang cells were mixed with the spleen effector cells and incubated at 37°C for 18 hr. For both assays, the supernatants in each well containing released 51Cr were collected and counted in a γ-counter. Spontaneous release was always less than 10% of total release obtained with 2% sodium dodecyl sulfate. NK activity and ADCC are expressed as:

\[
\text{% of cytotoxicity} = \frac{\text{Test cpm} - \text{spontaneous cpm}}{\text{cpm initially incorporated}} \times 100
\]

The tests were performed with 3 different effector/target ratios: (a) 50/1; (b) 100/1; and (c) 200/1. Only the results with the ratio 200/1 are given.

Lymphocytes Proliferation Assays. Viable spleen cells were cultured in 4 replicates in the round-bottomed wells of a microtiter plate (2.5 x 10^6 cells/well) in RPMI 1640 medium, supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 1% glutamine, and 10% heat-inactivated fetal bovine serum. Optimal concentrations of purified phytohemagglutinin (0.25 µg/ml), concanavalin A (0.5 µg/ml), or bacterial LPS (20 µg/ml) were added, and the cultures were incubated for 3 days at 37°C. Six hr prior to the culture termination, 1.0 µCi of [3H]thymidine (0.05 ml) was added to each culture. The cells were harvested with a multiple sample harvester onto the strips of fiberglass paper, and [3H]thymidine incorporation into DNA was determined by liquid scintillation spectroscopy. The background values were 700 ± 54 cpm (S.D.), and the data are expressed as net cpm (cpm in stimulated cultures minus cpm in cultures with no mitogen).

PFC Assay. Spleens were removed from mice sacrificed 4 days after i.v. injection of 5 x 10^6 (0.1 ml of 10% suspension) sheep RBC. Single-cell suspensions were tested in Cunningham’s modification of the Jerne plaque assay; a mixture of either 1 x 10^5 or 1 x 10^6 spleen cells (0.2 ml), 0.4 ml of sheep RBC, 0.1 ml of guinea pig complement, and 0.3 ml of medium was pipetted into Cunningham chambers. The chambers were sealed with petroleum jelly and were incubated at 37°C for 1 hr, and plaques were counted using an inverted microscope. The data were expressed as PFCs per spleen.

Tumor Studies In Vivo

Tumor Growth s.c. Tumor cells were harvested from exponentially growing cultures with mild trypsinization, washed twice in ice-cold medium without fetal calf serum, diluted in appropriate concentrations, and injected s.c. into the flanks of nude mice in 0.5-ml volumes. The mice were checked daily for tumor take, and when the tumors were growing, 2 perpendicular diameters were measured 2 to 3 times weekly, and mean tumor diameter for each group was calculated.

Assay for Growth of Experimental Pulmonary Metastases. The B16F10 cells were harvested from in vitro cultures as described above. Single cell suspensions of the LOX tumor were in this case obtained from s.c. xenografts growing in nude mice, removed under sterile conditions, cut into pieces of 4.5 mm, and mixed using a Stomacher 80 Lab-Blender, Seward Surgical, London, England. The cell suspension was passed through sterile gauze, and viable cells, determined by Trypan blue exclusion, were diluted in medium to appropriate concentrations and injected i.v. in 0.2-ml volumes. The mice were checked daily, and the survival time of mice that died was recorded. In some experiments, one-half of the animals within each group were killed on Day 25 (LOX) or Day 18 (B16F10) after tumor cell injection. The lungs were removed and fixed in Bouin’s solution, and the number of tumor colonies was counted.

RESULTS

Immune Characteristics of Different Nude Mouse Genotypes

Adult NIH nude mice are known to have high NK activity (19), whereas young (2 to 3 weeks old) nude mice have low NK activity (11, 13, 14). However, the results in Table 1 show that the young NIH nude mice gave lower values than adult NIH nude mice, not only in the NK test, but also in the assays for K-cell (ADCC), T-cell (phytohemagglutinin and concanavalin A), and B-cell (LPS) activity, as well as in T-dependent B-cell response (PFC) assay.

For the beige-nude mice, the NK activity was much lower than for adult NIH nude mice (Table 1). The difference is statistically significant in the Wilcoxon test (p < 0.01). The NK activity in beige-nude mice was also appreciably lower than in the “low NK” (14, 15, 18), 2- to 3-week-old NIH nude mice (Table 1). Compatible with the suggestion that a relationship may exist between the effector cells in the NK and ADCC assays (33, 40), the values in these tests were closely similar for beige as well as for NIH nude mice. The response to the T-mitogens was lower, although not significantly, in beige than in adult NIH mice, whereas the LPS response was similar in beige and in 2- to 3-week-old NIH nude mice, and the PFC values were similar in beige and in adult NIH animals.

The NIH-II mice have a defective B-cell response, due to the acquisition of the XID gene (1), as reflected here in the LPS and PFC results (Table 1). In contrast to the situation in the other variants of nude mice, the activity in NIH-II mice of K-cells, as
reflected in the ADCC test, was low compared to the NK activity. The PHA and concanavalin A responses were similar in NIH-II and in adult NIH nude mice.

Tumor Studies

Take Rate and Growth Rate of Tumors Inoculated s.c. Table 2 shows the take and growth rates of tumors inoculated s.c. into mice belonging to the different groups. Surprisingly, the human tumors, LOX, a melanoma, the undifferentiated T-cell lymphoma, CEM, and the chronic myelogenous leukemia, K562, did grow equally well in adult NIH nude mice, with high NK activity, and in beige-nude mice, with low levels of NK activity. Furthermore, the only 2 mice in which the K562 cells formed tumors, were both adult, "high NK" NIH mice. For the CEM and K562 tumors, the tumor cell inoculum was close to the limit of tumor take, whereas, for the LOX tumor, it was approximately twice the limit. The take and growth rates of these tumors were not significantly better in NIH than in beige nude. However, in contrast to what would be expected, it is clear that the growth was not favored in the low-NK beige animals.

Whereas the K562 cells are known to be moderately sensitive to mouse NK cells (16), it is conceivable that, due to species specificity, the other 2 human lines may be resistant to murine NK cells. Therefore, we also studied, in all 4 groups of mice, the s.c. growth of YAC-1 cells, i.e., the NK-sensitive cells (16, 26) that were used as targets in the in vitro NK cytotoxicity test. It is seen (Table 2) that when a high number of tumor cells (5 x 10^5) was injected, tumors developed in all animals, but the time to reach a mean tumor diameter of 10 mm was longer in beige than in adult NIH mice. When the challenge was reduced to 5 x 10^4 cells, near the limit of tumor take (26), both the take rates and growth rates of the YAC-1 tumors were reduced in beige and NIH-II mice compared to the results in young and adult NIH nude mice. Thus, in contrast to data published previously (10), an inverse correlation was not seen between s.c. tumor growth in vivo and the NK activity measured in vitro.

Progressive Growth of Pulmonary Metastases. From studies in mice, it has been concluded that NK cells may participate in the control of metastasis formation (11, 13, 15, 28, 36). To test this hypothesis, we studied 2 melanoma cell lines, the human, LOX (8), and the murine, B16F10 (7), which both have a high lung colonization capacity upon i.v. injection (7, 8, 16). If NK cells indeed are involved in preventing tumor cells from forming metastases, it would be expected that after i.v. injection of these tumor cells, the beige nude mice would get more lung tumors and/or die earlier than animals in the other groups.

Since, in separate experiments, a correlation was found between the number of lung colonies, present 25 (LOX) or 18 (B16F10) days after tumor cell injection, and the life span of mice that were let to die of their lung tumors (data not shown), the latter was chosen as end point for these experiments.

The experiments with the LOX cells showed that the fraction of animals that died of lung tumors after injection of 1 x 10^6 cells was in fact lower in the beige mice than in mice belonging to the groups with much higher NK activity (Table 3). Furthermore, the survival time of those animals that died was not shorter for beige mice than for the other groups, if anything, it was longer. Also, in spite of the immunological differences between young and adult NIH mice, the fraction of mice dying and their survival time were the same in the 2 groups. The fraction of NIH-II mice that died was lower than in young and adult NIH animals, but higher than in beige-nude mice, whereas the life span was the same as for the NIH mice (Table 3).

Experiments with B16F10 cells, previously shown to be NK sensitive in vitro (15), were carried out as described by Hanna and Fidler (14, 15), except that mouse life span was used as end point instead of counting lung colonies. After i.v. injection of 5 x 10^6 tumor cells, all animals, except 2 of 17 NIH-II mice, died within 3 to 5 weeks (Table 3). However, in contrast to the
Table 3
Lethality of nude mice given i.v. injections of human (LOX) and murine (B16F10) melanoma cells

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of cells injected</th>
<th>NIH (6 wk old)</th>
<th>NIH (2-3 wk old)</th>
<th>Beige</th>
<th>NIH II</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX</td>
<td>$1 \times 10^6$</td>
<td>48/55 (1.0)</td>
<td>11/12 (1.0)</td>
<td>5/17  (1.2)</td>
<td>9/15  (1.0)</td>
</tr>
<tr>
<td>B16F10</td>
<td>$5 \times 10^4$</td>
<td>20/20 (1.0)</td>
<td>15/15 (0.96)</td>
<td>17/17 (0.96)</td>
<td>15/17 (1.2)</td>
</tr>
</tbody>
</table>

* The ratio of mice that died of lung colonies per total number of mice given injections.

Numbers in parentheses, mean survival time of mice in each group, normalized to that of 6-week-old NIH nude mice. This was 38 days for mice given injections of LOX cells and 27 days for mice given injections of B16F10 cells.

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The end points used here for assessment of tumor growth do not necessarily reflect only specific interactions between tumor cells and NK cells. The rapid elimination of tumor cells observed from lungs, liver, and spleen of mice is, however, generally attributed to NK cells (11, 20, 32). Therefore, we injected $^{125}$I(Urd-labeled tumor cells i.v. into mice of the beige and the 2 NIH categories. At different periods of time after tumor cell injection, animals were killed, and the amount of radioactivity retained in the tissues was measured. Control experiments showed that the labeling procedure did not affect the viability of the tumor cells, as it was found that mice given injections of radiolabeled tumor cells had the same life span as mice receiving unlabeled cells.

Of the beige mice, which is consistent with the results of the life span studies (Table 3). In liver and spleen, the radioactivity disappeared at the same rate in the 3 groups (data not shown).

With labeled B16F10 cells, no significant difference in tumor cell retention was seen between the groups, neither in the lung (Chart 1B) nor in the other tissues. Even though the same tumor line was used, the results do not confirm data previously obtained in young and adult nude mice (13–15). Furthermore, in the present study, the NK-sensitive B16F10 cells (15) were eliminated as rapidly from the lungs of the low NK beige mice as from NIH mice with high NK levels.

The NK cell activity in vitro is measured by testing the cytotoxicity of mouse spleen cells against YAC-1 tumor cells. In an in vivo experiment resembling the in vitro test, we injected labeled YAC-1 cells into mice and measured the radioactivity retained in the spleen. No difference between the 3 groups was observed (Chart 1C). Similarly, no clear difference in retained radioactivity was seen in lung and liver.

DISCUSSION

The great majority of published reports on the relationship between NK cell activity, tested in vitro, and in vivo tumor growth control, suggests that NK cells are important in primary host defense against tumors (12, 14, 15, 18, 19, 23, 36, 38). The
present results do not support this notion. Thus, in 4 variants of nude mice, low NK activity was not associated with increased growth of s.c.-inoculated human (LOX, CEM, K562) and murine (YAC-1) tumor cells, nor was low NK activity associated with increased lung colony formation in a metastasis model using i.v.-injected human (LOX) and murine (B16F10) melanoma cells. Moreover, there was no relationship between host NK activity and the rate of elimination of 125IIdUrd-labeled LOX, B16F10, and YAC-1 cells from lungs, liver, or spleen.

In several of the previous reports (10, 15, 22, 23, 36), the selectivity of the NK impairment was not carefully investigated, and immune differences other than in NK activity may have influenced the results. This could be the case when increased tumor growth in young, compared to adult, NIH nude mice was ascribed to differences in NK activity, and considered as evidence for the in vivo significance of NK cells (11, 13, 14). Thus, in light of the other immunological differences demonstrated here (Table 1), this conclusion may not be valid.

Beige-nude mice with low NK activity have previously been suggested to be a valuable tool for studies of tumor growth and metastasis, and for evaluating the in vivo role of NK cells in immune surveillance against tumors (5, 22). However, until now such studies have not been reported, possibly due to problems with the viability of the mice (22). Our beige-nudes were healthy and had a life span similar to that of regular nude mice. Through a comparison of the immune status of both parental strains, and as shown in Table 1, it was established that the beige-nude mice had immune characteristics that were promising for such tumor experiments.

To our surprise, the growth of the human and mouse tumors tested was not increased in these low-NK mice compared to nude mice with high-NK activity. Beige mice are not completely devoid of NK cells, but the differences in NK function between the different types of nude mice here tested were highly significant, and the present results strongly question the view that NK cells have important cytotoxic effects in vivo. It is well-known that tumor inoculations, as well as interferon and the copolymer of polyinosinic and polycytidylic acids, can induce a transient increase in NK activity, and this increase could theoretically be different in different nude strains. It seems reasonable to assume, however, that the magnitude of this response will not be larger in beige-nude mice with deficient NK function than in mice of genotypes with normal or high initial NK values.

The different nude genotypes examined did not differ in NK function only (Table 1). However, the other immune defects found would be expected, if anything, to favor tumor growth in the beige-nude animals. Macrophage function was not tested, and since macrophages may be involved in natural resistance to tumors (6, 35), possible differences in macrophage activity between the mouse genotypes could perhaps influence tumor growth and help to explain the present data. However, it is known that the macrophages in regular beige mice show abnormal granules, defective chemotaxis, and delayed antitumor activity in vitro (31), and it is likely that this is the case also in beige nude mice. If so, this would be thought to favor, not suppress, tumor take and growth in the beige animals.

It is well-known that host immune response may easily be affected by viral infections in the animals. Therefore, it should be emphasized that no virus infection was detected during the present studies, a fact probably reflected by the overall relatively low NK values (14, 15, 18) for the different groups of mice (Table 1).

The B-cell-deficient NIH-II nude mice had much lower values in the ADCC assay than in the NK test. This finding is not consistent with the view that the NK and K-effector cells are the same (33, 34, 41). NIH-II mice might, therefore, be useful in studying the lineage of the K-cells, and the results raise the question of whether K-cell function may depend partly on a normal B-cell activity.

No simple answer is available to the question of why the present data are inconsistent with previous results (11, 12, 15, 23, 24, 36) on the in vivo role of NK cells. If NK cells indeed have an important role in vivo, one would have expected to find differences between the mice in take and growth rates of s.c.-inoculated tumors (10). Furthermore, the claim that NK cells are important in preventing metastasis formation (11, 13, 15, 28, 36) was not supported by the results of our lung colony experiments with the human LOX and the murine B16F10 melanomas. In the present study, life span was used as end point, whereas Hanna and Fidler (15), in similar experiments with the B16F10 tumor, counted the number of lung colonies present 3 to 5 weeks after tumor cell injection. The use of different end points cannot explain the discrepancy between the results, as, in our experiments, a correlation was found between the number of lung colonies and the life span of the animals. It may be of importance, however, that whereas Hanna and Fidler (15) reported that adult, regular nude mice had developed very few lung colonies 3 to 5 weeks after injection of 5 x 10⁴ B16F10 cells (15), a high number of pulmonary metastases was found at Day 18 in our adult NIH nude mice, which started to die of lung tumors approximately 3 weeks after injection of the cells. The beige-nude mice used in our experiments do in part have the C57BL genetic background, and since the B16F10 tumor is syngeneic for C57BL (7), the B16F10 cells could possibly have a growth advantage in the beige-nude mice. Nevertheless, no difference in lung colony growth between beige and NIH nude mice was seen.

In contrast to the results of previous studies on B16F10 cells (15), a lack of relationship was found here between host NK activity and the rate of elimination of 125IIdUrd-labeled tumor cells from lungs, liver or spleen. However, since consistent results were obtained with all 3 tumors studied, our data strongly indicate that NK cells do not have an important role in early host defense against tumor cells.

In agreement with data recently obtained in conventional mice (35), the overall conclusion of the present study is that, although NK cells clearly are cytotoxic in vitro, they do not seem to exert any significant direct effect on tumor cells in vivo. The mechanisms involved in host defense against tumors are apparently more complex than previously appreciated. Thus, the demonstrated differences in tumor growth control between the 4 types of mice cannot easily be explained on the basis of the immunological data. It should be noted, however, that in all strains of nude mice tested, minor responses to the T-cell mitogens were seen (Table 1). It is possible that nude mice have T-like cells (27, 30) that may become functional after induction (9, 21, 37). The distribution of such cells may differ between the nude genotypes. Also, it is conceivable that interactions between different immune cells (20, 39, 41), possibly including NK cells, or factors produced by them (9, 17, 25), are important in the regulation of the immune response (3). Therefore, it seems reasonable to suggest that,
rather than attempting to explain tumor growth data in vivo by immunological differences restricted to one single parameter, the interaction between different immune cells should be further investigated. In such studies, nude mice with various combinations of immune defects may prove valuable.

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