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Lysis of fresh murine mammary tumor cells by syngeneic natural killer cells and lymphokine-activated killer cells

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Summary. We have compared the ability of natural killer (NK) cells from two substrains of C3H mice that differ with respect to their susceptibility to the development of mammary adenocarcinomas to lyse fresh syngeneic mammary tumor cells. Single cell suspensions of mammary tumors from retired breeder females were used as targets in 22-h ^{51}Cr-release cytotoxicity assays with syngeneic NK cells. Tumor cell suspensions were prepared by enzymatic digestion of finely minced tissue followed by centrifugation through a discontinuous Percoll gradient. Effector cells were prepared by passing spleen cells over nylon wool followed by centrifugation through Percoll fraction 7. Syngeneic NK cells had significant levels of lysis against 5/8 tumors studied. NK cells from low risk animals (C3Heb/FeJ) consistently demonstrated greater cytotoxicity against tumor cell preparations than did effectors from the high tumor substrain (C3H/OuJ). Study of cytocentrifuge preparations stained with Wright-Giemsa revealed that the two substrains were identical with respect to the number of azurophilic granules present in the cytoplasm of their NK cells. We have also shown that lymphokine-activated killer (LAK) cells can be generated from splenocytes in C3H mice. While LAK cells from both substrains were capable of lysing fresh syngeneic mammary tumor cells in vitro, LAK cells from the animals at high risk for the formation of mammary adenocarcinomas had greater cytotoxicity against tumor cell suspensions than LAK cells from the low tumor substrain.

Introduction

Considerable attention is being focused on the role of natural killer (NK) cells in immunosurveillance against neoplastic cells, and it has been suggested that they may be part of the first line of defense against malignant growth [25]. The evidence supporting the conclusion that NK cells are responsible for rejection of implanted syngeneic tumors and inhibition of experimental tumor metastasis is extensive [54]. However, much less work has been reported on the role of these cells in resisting the growth and metastasis of induced or spontaneous primary tumors. A major problem in this respect has been the lack of a proper model system. As noted by Stutman [50], such a system should involve animals with the same genetic background that differ in their levels of NK cell activity. It appears that the C3H mouse may provide that system. It is felt that mice of the C3H/OuJ and C3Heb/FeJ substrains are genetically identical, since they do not differ for any of the genetic markers that have been studied, including histocompatibility [21].

In an effort to determine whether NK cells play a role in resisting the development of primary tumors we confirmed that these two substrains differ with respect to their susceptibility to the development of mammary tumors, and we demonstrated that they also differ in their levels of NK cell activity [1]. Animals of the C3H/OuJ substrain are at high risk for the formation of mammary adenocarcinomas, while C3Heb/FeJ mice have a low incidence of such tumors. Natural cytotoxicity of splenic mononuclear cells was lower in the high risk substrain, suggesting that a lesion in NK cell activity may be involved in murine mammary tumorigenesis. As we continue to develop the C3H mouse mammary tumor system as a model for studying the role of NK cells in controlling primary tumors, a number of questions remain to be answered. Some of these are as follows: (1) are NK cells from both substrains capable of lysing fresh syngeneic mammary tumor cells in vitro? (2) Is there a difference in the ability of NK cells from the high and low risk animals to lyse the tumor cells? (3) Do NK cells from the two substrains differ with respect to the number of azurophilic granules present in their cytoplasm? (4) Can lymphokine-activated killer (LAK) cells be generated in C3H mice? (5) Are LAK cells from both substrains capable of lysing fresh syngeneic mammary tumor cells in vitro? (6) Is there a difference in the ability of LAK cells from the low and high risk animals to lyse the tumor cells? We addressed these issues in the present investigation.

Materials and methods

Animals. Mice of the C3H/OuJ substrain are characterized by the presence of mouse mammary tumor virus (MMTV) and are at high risk for the development of mammary adenocarcinomas. This substrain was isolated from the C3H/HeJ stock. Mice of the C3Heb/FeJ substrain do not carry MMTV and have a low incidence of mammary tumors. This substrain was developed by transplantation of C3H/HeJ ovaries into C57BL/6 female recipients subsequently

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mated to C3H/HeJ males [21]. All animals were purchased from the Jackson Laboratory, Bar Harbor, Me., at 4–5 weeks of age. They were healthy upon arrival and remained disease-free for the duration of the experiments. Mice were maintained under uniform conditions in the animal quarters at Health Science Center at Syracuse, and they were used for the preparation of effector cells when they were between 6 and 12 weeks of age.

Tumor target cells. YAC-1, a tissue culture cell line derived from a Moloney virus-induced lymphoma in A/Sn mice [7], was maintained as an in vitro suspension culture in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamicin. For the evaluation of NK activity, 10×10^6 YAC-1 cells were labeled with 200 μCi sodium ^{51}chromate (250–500 mCi/mg Cr; Amersham Corp. Arlington Heights, Ill.) as previously described [1].

Primary spontaneous mammary tumors were obtained from ether-anesthetized 8 to 10-month-old C3H/OuJ retired breeder mice. Tumors were excised and rinsed with Hanks' balanced salt solution (HBSS). Subsequent to the removal of blood clots and necrotic areas, tumor tissue was minced with scissors. Cell suspensions were prepared by treatment of the finely minced tumor tissue with collagenase (297 units/ml), trypsin (2 mg/ml), and DNase (0.2 mg/ml) in HBSS with 10 m*M* HEPES buffer adjusted to pH 7.3 for 90 min at 37° C in a shaking water bath. The resulting cell suspension was passed through stainless steel mesh, and the cells collected by centrifugation, washed, and resuspended in 1.0 ml RPMI 1640 with 10% FBS. Cell separation was achieved by centrifugation through a discontinuous Percoll (Pharmacia Chemicals, Uppsala, Sweden) gradient. The gradient was composed of 2.5 ml layers of 66.7%, 40.8%, 35.4%, 30.0%, and 20.0% Percoll. Cells from the five bands were collected from the top with a Pasteur pipet, washed twice with RPMI 1640 with 10% FBS and examined for viability by means of trypan blue exclusion. Experiments were performed only with suspensions that were at least 75% viable. The cellular composition of each fraction was studied in cytocentrifuge preparations stained with Wright-Giemsa, and it was determined that band three contained the highest percentage of tumor cells in single cell suspension. Mammary tumor cells were collected by centrifugation and resuspended in 0.2 ml phosphate-buffered Ringer's (PBR) containing 10% FBS. They were labeled by incubation with 400 μCi sodium ^{51}chromate (250–500 mCi/mg Cr; Amersham Corp.) for 90 min at 37° C. The labeled cells were washed three times with PBR and resuspended at a concentration of 0.2×10^6 cells/ml in RPMI 1640 medium supplemented with 40% FBS.

Preparation of NK cells. Spleens were obtained from ether-anesthetized animals, and single cell suspensions were prepared by forcing the tissue through a stainless steel wire mesh. Pooled cells from three animals were used in each experiment. Adherent cells were removed by incubating whole spleen cells for 45 min at 37° C on columns of 4 g of nylon wool in 30-ml plastic disposable syringes [27]. The nonadherent cells were eluted in 50 ml of warm RPMI 1640 medium supplemented with 10% FBS and further purified by centrifugation through Percoll fraction 7. Cells from the interface between layers were collected, washed three times, and resuspended in RPMI 1640 medium [53].

Cytotoxicity assay. A standard ^{51}Cr-release cytotoxicity assay in 96-well, round-bottomed microtiter plates (Linbro Scientific Co., McLean, Va.) was used [1]. In experiments designed to study NK cell activity, plates were incubated for 22 h at 37° C in a humidified 5% CO_2 atmosphere, while in assays for LAK cell activity the incubation period was 18 h. Spontaneous release of isotope was estimated by incubating the labeled target cells alone. Maximal isotope release was estimated by exposure of labeled target cells to 10% Triton X-100. All determinations were performed in triplicate, and percent cytotoxicity was calculated as:

$$\% \text{ cytotoxicity} = \frac{\text{cpm of test group} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100$$

Evaluation of cell morphology. The morphology of effector cell preparations was evaluated by microscopic analysis of Wright-Giemsa-stained cytocentrifuge preparations. At least 100 cells were analyzed from each slide.

Generation of LAK cells. Spleens were obtained from ether-anesthetized animals, and single cell suspensions were prepared by pressing the tissue through fine stainless steel mesh into RPMI 1640 medium supplemented with 10% FBS. The cells were pelleted by centrifugation, resuspended in 2.0 ml RPMI 1640 containing 10% FBS, and placed above 2.0 ml Percoll fraction 7. Tubes were centrifuged for 30 min at 550 xg, and cells from the interface between layers were collected, washed with RPMI 1640 medium and resuspended in LAK medium consisting of RPMI 1640 supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (0.1 μg/ml), gentamicin (50 μg/ml), 5×10^{-5} *M* 2-mercaptoethanol, and 20 m*M* HEPES buffer. LAK cells were generated by placing 2.7×10^7 splenocytes into tissue culture flasks (No. 25100; Corning Glass Works, Corning, NY) in 9 ml of LAK medium containing 4,500 units recombinant interleukin-2 (rIL-2; Hoffmann-LaRoche, Nutley, NJ). The flasks were incubated at 37° C in a humidified 5% CO_2 atmosphere for 72 h. LAK cells were harvested, washed three times, and resuspended in RPMI 1640 medium. Fresh splenocytes and splenocytes cultured for 3 days in the absence of rIL-2 were used as controls.

Results

The NK cells from both substrains were tested for their ability to lyse fresh syngeneic mammary tumor cells in 22-h ^{51}Cr-release cytotoxicity assays. It is evident from the data presented in Table 1 that syngeneic NK cells had significant levels of cytolytic activity against five of the eight tumors studied. In a representative experiment with effectors from the low tumor substrain (C3Heb/FeJ), the cytotoxicity was 83.2%, 59.9%, and 46.8% for effector:target (E:T) ratios of 80:1, 40:1, and 20:1, respectively. Comparable values obtained with NK cells from the high risk substrain (C3H/OuJ) were 59.9%, 36.1%, and 17.5%. The NK cells from low risk animals consistently demonstrated greater cytotoxicity against tumor cell preparations than effectors from the high tumor substrain. The data shown in Table 1 were tested for statistical significance using the paired Student's *t*-test [49] which confirmed that the difference between the substrains in their ability to lyse fresh syngeneic mammary tumor cells in vitro was significant at all E:T ratios studied ($P < 0.05$).

Table 1. Natural killer cell activity against mammary tumor cells of splenic lymphocytes from C3H mice at low and high risk for the development of mammary tumors

	% Cytotoxicity					
	C3Heb/FeJ (low)			C3H/OuJ (high)		
Exp. #	80 : 1	40 : 1	20 : 1	80 : 1	40 : 1	20 : 1
1	71.6	44.6	ND[a]	46.8	27.7	ND
2		No lysis			No lysis	
3		No lysis			No lysis	
4	40.0	ND	15.3	7.0	ND	7.1
5	97.0	75.7	58.9	ND	37.5	18.4
6	83.2	59.9	46.8	59.9	36.1	17.5
7	119.6	100.1	43.1	8.0	3.2	5.2
8		No lysis			No lysis	
Mean	82.3	70.1	41.0	30.4	26.1	12.1
Standard error of the mean	13.3	11.9	9.2	13.5	7.9	3.4

[a] ND, not determined

We reported that C3H mice that are at high risk for the formation of mammary tumors are characterized by a lesion in NK cell activity [1], and we began to look for the nature of this lesion. Neither the number of large granular lymphocytes (LGLs) present in the cytotoxicity assays nor the ability of lymphocytes from the two substrains to bind to target cells could explain the difference in the level of NK cell activity between them. This suggested that the lesion in cell-mediated cytotoxicity in C3H/OuJ mice might involve the ability of their NK cells to complete the stages of the lytic sequence. Since NK cell-mediated cytolysis seems to be based on a granule exocytosis mechanism, we attempted to determine whether NK cells from the two substrains differ with respect to the number of azurophilic granules residing in their cytoplasm. To this end, morphological studies of LGLs were performed by microscopic analysis of Wright-Giemsa-stained cytocentrifuge preparations. Considerable variation in granule number was observed; the range in number of granules per cell extended from 1 to 27 for the 1000 cells examined. However, the mean number of azurophilic granules per LGL was 7.2 for both substrains.

Incubation of normal murine splenocytes in the presence of the lymphokine IL-2 resulted in the generation of lymphoid cells with the ability to lyse fresh NK cell-resistant target cells in vitro [42]. A variety of cancer cells have been shown to be susceptible to lysis by these LAK cells, but mammary adenocarcinoma cells have infrequently been used as targets in this work. For this reason, we generated LAK cells from C3Heb/FeJ and C3H/OuJ mice and studied their ability to lyse fresh syngeneic mammary tumor cells in ^{51}Cr-release cytotoxicity assays.

A series of experiments was performed in an effort to determine the optimal concentration of rIL-2 to include in the culture medium. With YAC-1 cells as targets in 4-h ^{51}Cr-release cytotoxicity assays, rIL-2 at a concentration of 250 units/ml produced a satisfactory level of cytotoxicity. In a representative experiment with LAK cells from the low tumor substrain, the cytotoxicity was 72.1%, 64.3%, and 51,1% for E:T ratios of 80:1, 40:1, and 20:1, respectively. Comparable values obtained with fresh splenocytes from the low risk substrain were 24.3%, 16.7%, and 10.0%. Therefore, exposure to rIL-2 at a concentration of 250 units/ml for 72 h enhanced the cytolytic activity of splenocytes at least 3-fold.

With mammary tumor cells as targets, we found it necessary to increase both the concentration of rIL-2 to 500 units/ml and the duration of the assay to 16–18 h in order to achieve a satisfactory and reproducible level of cytotoxicity. Table 2 presents a summary of the results of five experiments in which we studied the ability of LAK cells generated from both strains of C3H mice to lyse YAC-1 and fresh syngeneic mammary tumor cells in 18-h ^{51}Cr-release cytotoxicity assays. YAC-1 cells were included as targets in this phase of the investigation in order to monitor the LAK cell generating system. It is clear from the data

Table 2. Lymphokine-activated killer cell activity of splenic lymphocytes from C3H mice at low and high risk for the development of mammary tumors

		% Cytotoxicity[a]											
		Against YAC-1 cells						Against mammary tumor cells					
		C3Heb/FeJ			C3H/OuJ			C3Heb/FeJ			C3H/OuJ		
		Effector: target ratios						Effector: target ratios					
		80 : 1	40 : 1	20 : 1	80 : 1	40 : 1	20 : 1	80 : 1	40 : 1	20 : 1	80 : 1	40 : 1	20 : 1
Effector cells	Interleukin-2												
Fresh splenocytes	0	52.0 (5.8)	36.4 (5.1)	21.4 (5.2)	56.1 (5.4)	41.8 (5.7)	22.2 (5.0)		No lysis			No lysis	
Cultured splenocytes	0		No lysis			No lysis			No lysis			No lysis	
Cultured splenocytes	500 units	91.0 (5.2)	90.9 (3.5)	68.1 (3.6)	94.5 (5.1)	93.8 (2.6)	85.9 (3.7)	25.9 (3.3)	19.5 (4.9)	14.4 (4.6)	40.6 (2.9)	31.9 (0.4)	22.5 (2.5)

[a] Each value represents the arithmetic mean of five independent experiments.
Standard errors of the mean are shown in parentheses

Table 3. Cellular composition of final effector cell preparations

	C3Heb/FeJ Fresh	C3H/OuJ Fresh	C3Heb/FeJ[a] Cultured −	C3H/OuJ Cultured −	C3Heb/FeJ[b] Cultured +	C3H/OuJ Cultured +
Small to medium lymphocytes	60.6 ± 2.6[c]	56.6 ± 2.2	69.6 ± 1.1	66.7 ± 1.6	63.5 ± 4.0	62.2 ± 5.1
Large agranular lymphocytes	11.0 ± 2.9	11.2 ± 3.4	8.9 ± 0.3	9.4 ± 2.3	8.5 ± 0.7	6.7 ± 1.8
Large granular lymphocytes	9.0 ± 1.6	7.1 ± 0.8	1.3 ± 0.7	1.2 ± 0.3	6.2 ± 2.2	4.7 ± 0.2
Blasts (agranular)	1.3 ± 0.4	1.7 ± 0.3	5.2 ± 0.3	5.0 ± 0.5	6.6 ± 1.3	8.1 ± 2.2
Blasts (granular)	0.00	0.00	0.3 ± 0.2	1.5 ± 1.0	5.9 ± 1.6	7.2 ± 0.8
Granulocytes	12.5 ± 1.5	13.6 ± 4.6	10.2 ± 1.6	12.7 ± 2.6	5.2 ± 0.5	9.2 ± 0.3
Others	5.3 ± 2.4	9.9 ± 3.8	4.1 ± 1.0	2.7 ± 1.3	4.0 ± 0.8	1.8 ± 0.3

[a] Splenocytes cultured for 3 days in the absence of recombinant interleukin − 2 (rIL-2)
[b] Splenocytes cultured for 3 days in the presence of rIL-2. (500 units/ml)
[c] Arithmetic mean ± standard error of the mean of percentages of cell types

that freshly prepared splenocytes from high and low risk animals demonstrated similar levels of cytotoxicity in vitro against YAC-1 cells. When these splenocytes were cultured for 3 days in the absence of rIL-2, they were no longer effective against this target. However, after culture for 3 days in the presence of rIL-2 at a concentration of 500 units/ml, the cytolytic activity against YAC-1 cells was markedly enhanced. Although the concentration of rIL-2 employed and the duration of the assay (18 h) were not optimal for measuring cytotoxicity against the YAC-1 target, splenocytes cultured in the presence of rIL-2 showed a 3 to 4-fold increase in their ability to lyse these cells at an E:T ratio of 20:1. It is interesting that at this E:T ratio the cytolytic activity of LAK cells from the animals at high risk for the development of mammary adenocarcinomas (85.8%) was greater than that of LAK cells from the low risk substrain (68.1%). Neither fresh splenocytes nor splenocytes cultured for 3 days without rIL-2 showed any cytolytic activity against fresh syngeneic mammary tumor cells. However, splenocytes cultured for 3 days with rIL-2 were fully capable of lysing these tumor cell targets. It is interesting that LAK cells from high risk animals consistently demonstrated greater cytotoxicity against tumor cell preparations than effectors from the low tumor substrain. However, when the data were tested for statistical significance by the paired Student's *t*-test [49], the difference between the substrains with respect to the ability of their LAK cells to lyse fresh syngeneic mammary tumor cells in vitro was only found to be significant ($P < 0.05$) at the E:T ratio of 80:1.

The morphology of the final preparations of fresh splenocytes and splenocytes cultured for 3 days in the presence and absence of rIL-2 was evaluated by microscopic analysis, and the cellular composition of these effector cell preparations is shown in Table 3. One can see that the numbers of small to medium lymphocytes and large agranular lymphocytes were about the same in all preparations. On the other hand, LGLs decreased in number in splenocytes cultured without rIL-2, while they were maintained fairly well in splenocytes cultured with rIL-2. Few lymphoblasts were observed in fresh splenocytes. However, they increased in splenocytes cultured without rIL-2, and they increased further in those exposed to rIL-2. Furthermore, a dramatic increase in the number of lymphoblasts containing azurophilic cytoplasmic granules was observed in the LAK cell preparations.

Discussion

The role of the immune system in resisting the growth and metastasis of tumors is the subject of intense investigation, and the concept of immune surveillance has received considerable experimental support. For a while the cytotoxic T cell received most of the attention, but recently other cell types have been emerging as alternatives to T cell-mediated tumor resistance. One of these is the NK cell, a heterogeneous population of normal lymphoid cells [36, 60] that have spontaneous cytotoxic activity against malignant cells, virus-infected cells, and some normal cells [24, 25].

The role of NK cells in the in vivo resistance against established tumor cell lines has been demonstrated. The major experimental approach in this work has been to attempt to correlate in vivo resistance to tumor cell transplants with the level of NK activity in the recipient animals, and indeed a good correlation has been observed by several investigators [24, 28, 41]. The transfer of increased resistance to tumor growth as well as increased clearance of inoculated labeled tumor cells by means of transfer of NK cell-enriched populations or of bone marrow precursors of NK cells has also been demonstrated [6, 18, 29, 40]. In addition, NK cells have been shown to play a role in inhibiting the metastasis of transplantable tumors [19, 20, 52]. Another approach has been to study both the resistance of transformed cell lines to NK-mediated cytotoxicity in vitro and their tumorigenic potential in vivo. In the case of adenovirus 2-transformed rat embryo fibroblast cell lines, it has been shown that there is a direct relationship between these two parameters [48].

Such results, however, give no indication of whether NK cells play a similar role in the defense against growth and metastasis of spontaneous or carcinogen-induced primary tumors. This is a fundamental but more difficult question to answer, and most of the available evidence is suggestive. As noted by Herberman [22] this includes the low incidence of spontaneous tumors in animals with high NK activity, the high incidence of lymphoproliferative disease in humans and animals with low NK activity, the enhancement of NK activity by retinoic acid, an agent that retards tumor development, and the inhibition of NK activity by some carcinogens or tumor promoters.

It has been suggested [23] that in order to more definitively answer the above question, we must be able to demonstrate that primary tumor cells are susceptible to recog-

nition and lysis by NK cells, that NK cells from tumor-bearers are able to interact with autologous tumor cells, and that selective alterations in NK activity in tumor-bearing individuals can affect the growth or metastasis of the tumors. Some experimental evidence has been published on each of these points. Murine nonlymphoid tumor cells harvested directly from in vivo tumors, as well as lymphomas, are susceptible to lysis by NK cells [35]. Some freshly prepared human tumor cells have also been significantly lysed by NK cells [43, 47, 55, 57, 61]. One line of evidence that supports the possibility that NK cells are able to interact with autologous primary tumor cells is the observation that they can enter and accumulate at the site of tumor growth in small spontaneous mouse mammary carcinomas [11] as well as in small primary mouse tumors induced by murine sarcoma virus [4, 11]. More direct evidence on this point comes from the work of Uchida and his colleagues, who first demonstrated that cytotoxic potential for autologous tumor cells is present in the peripheral blood and pleural effusions of cancer patients. They found that cytotoxic activity is strongly associated with a minor proportion of LGLs and restricted to the cell population that can lyse NK-sensitive K562 cells [55]. Using a two-target conjugate cytotoxicity assay, they showed that a single effector cell can lyse both autologous tumor cells and K562 cells [56]. This provided direct evidence for the involvement of NK cells in autologous tumor cell killing. More recently, it was observed that lymphoma cells were also lysed by autologous NK cells [38].

Several investigators have utilized anti-asialo GM_1 serum, which reduces NK cell activity, to study the role of these cells in the defense against circulating tumor cells and the development of metastases. For example, Gorelik et al. [12] showed that it reduced the ability of mice to eliminate tumor cells that had been inoculated into the blood and enhanced the growth of artificial or spontaneous metastases. Barlozzari et al. [2] demonstrated that it caused a depression in in vivo clearance of tumor cells in rats. They further showed that this effect could be partially restored by adoptive transfer of relatively low numbers of highly enriched LGLs from peripheral blood [3]. Habu et al. [17] observed a good correlation between reduced NK activity due to anti-asialo GM_1 serum and enhancement of transplanted tumor growth in nude mice. The effect of poly I:C, which augments NK cell activity, on growth of transplantable tumors and the formation of metastases has also been studied. It has been shown that growth of a transplanted bladder tumor in mice was inhibited by poly I:C treatment [9, 10]. Gorelik et al. [13] also reported that pretreatment of mice with poly I:C had an antimetastatic effect.

Even less information is available about the role of NK cells in immunosurveillance against the initial development of spontaneous or carcinogen-induced tumors. The major reason for this has been the lack of an adequate model system. It has been reported that interferon or poly I:C treatment initiated early in life could retard the appearance of spontaneous mammary carcinomas in RIII mice [5] and that administration of poly A:U to newborn C3H/He mice had a prophylactic effect on the subsequent development of mammary tumors [31]. In neither case, however, was the effect of treatment on NK activity studied. It has also been shown that splenic mononuclear cells from C3H/He mice that had been inoculated with poly A:U had enhanced cytotoxic activity [58] and that the growth of chemically induced tumors was enhanced in NK-function-abolished mice [17]. In addition, an inverse relationship between familial incidence of cancer and NK activity has been demonstrated [51], suggesting that a lesion in natural cell-mediated cytotoxicity preexists in individuals at risk for the development of cancer. We have found that this phenomenon also occurs in C3H mice [1]. Furthermore, the defect in NK cell activity can be observed throughout most of the life-span of animals having a high risk of mammary cancer. This suggests that the C3H mouse may provide a useful system for studying the role of NK cells in resisting the development of primary tumors. Such a system should involve animals with the same genetic background that differ in their levels of NK cell activity [50]. The available evidence supports the conclusion that C3H/OuJ and C3Heb/FeJ mice are genetically identical. No differences have been demonstrated between them for any of the genetic markers that have been studied [21]. In addition, the observation that splenocytes from the low and high risk animals failed to respond to each other in mixed lymphocyte cultures while they both proliferated in the presence of C57BL/6 stimulator cells (our unpublished data) supports the conclusion that the two substrains are immunogenetically identical.

In our previous work with this system, we used YAC-1, a cell line derived from a Moloney virus-induced lymphoma in A/Sn mice [7], as the target cell in cytotoxicity assays [1]. It was important for us to determine whether NK cells from both substrains are capable of lysing fresh syngeneic mammary tumor cells in vitro. Serrate and Herberman [46] reported in abstract form only that spleen cells from C3H/HeN mice had low but significant levels of cytolysis against freshly harvested cells from 62% of mammary adenocarcinomas studied. Our observation of significant levels of lysis against five of the eight tumor cell preparations studied is in good agreement with their data. We also found that NK cells from low risk animals had greater cytotoxicity against mammary tumor cell preparations than effectors from the high tumor substrain. This was essentially what we observed when YAC-1 cells were the targets, and again it suggests that a lesion in NK cell activity may be involved in murine mammary tumorigenesis.

We previously examined two of the possible explanations for the observed difference in the level of NK cell activity in high and low risk mice [1]. We studied the cellular composition of the effector cell preparations and determined that the substrains did not differ with regard to the number of LGLs present in the cytotoxicity assays. Next we performed conjugation assays in order to learn whether there was a difference in the ability of lymphocytes from the two substrains to bind to the target cells. In this case the results were surprising, since the binding capacity of splenic mononuclear cells was greater in the animals with lesser NK cell activity. Since it appears that the lesion in cell-mediated cytotoxicity in C3H/OuJ mice involves the ability of their NK cells to complete the stages of the lytic sequence, we have continued our attempts to define it. In the present investigation we examined two additional parameters. First, we studied the morphology of LGLs from both substrains and showed that they were identical with respect to the number of azurophilic cytoplasmic granules.

Second, we compared the response of lymphocytes from both substrains to IL-2. In 1980 Rosenberg's group

found that it was possible to generate lymphoid cells with the ability to lyse fresh, noncultured autologous primary and metastatic cancer cells by incubating normal murine splenocytes or human peripheral blood lymphocytes in this lymphokine [32, 59]. These cells were called LAK cells, and they have been extensively characterized in murine and human systems [15, 16, 45]. There is a great deal of interest in the LAK system because of its tumoricidal ability and the current availability of purified rIL-2 [44]. It is felt by some that LAK cells are distinct from both NK cells and cytotoxic T lymphocytes [14]. However, recent evidence seems to indicate that the LAK phenomenon is not mediated by a unique cell type, but is predominantly mediated by IL-2-activated NK cells [37, 39]. Interleukin-2 appears to act by increasing the cytolytic potential of NK cells and inducing their cytotoxicity against a broader range of target cells [37]. A number of malignant cells have been shown to be susceptible to lysis by LAK cells, but mammary adenocarcinoma cells seldom have been utilized as targets in this work. There is no evidence, that we are aware of, that fresh murine mammary tumor cells prepared from solid primary tumors are susceptible to killing by LAK cells. Lotze et al. [32] reported the lysis of fresh breast carcinoma preparations from two patients by LAK cells. However, the source of the tumor cells was ascites fluid in both instances. This is also the case for the breast cancer specimen used by Grimm et al. [15]. The ability to generate LAK cells in a variety of mouse strains, including C3H, was reported by Rosenstein et al. [45], but they did not use mammary tumor cells as targets in their work. A primary spontaneous mammary carcinoma obtained from a C3H mouse was shown to be sensitive to LAK cell lysis [30]. However, this tumor tissue was used after several subcutaneous passages. More recently, primary explants of spontaneous mammary tumors from $CD8F_1$ mice were shown to be susceptible to lysis by IL-2-stimulated lymphocytes [33]. It has also been reported that mouse and human mammary tumor tissue maintained in culture can be killed by LAK cells generated from tumor infiltrating lymphocytes [34]. Therefore, we attempted to determine whether LAK cells from high and low risk C3H mice were capable of lysing syngeneic mammary tumor cells freshly isolated from solid tumors. We demonstrated that splenocytes cultured for 3 days in the presence of rIL-2 were fully capable of lysing these tumor cell targets. It should be noted that LAK cells from the high risk substrain had greater cytotoxicity against mammary tumor cells than did LAK cells from the low tumor substrain. This result is the opposite of what we observed with respect to the ability of unactivated NK cells from these substrains to lyse both YAC-1 and mammary tumor cells, and it suggests that the lesion in NK activity in C3H/OuJ mice may be due to a defect in IL-2 production. Studies designed to test this hypothesis are in progress.

We demonstrated here and in previous work with lymphocyte fractions enriched for NK cells [1] that effector cells from the low risk substrain were more active than those from high risk animals in lysing both YAC-1 and mammary tumor cell targets. However, it is apparent from the data in Table 2 that there was no difference in the ability of unfractionated fresh splenocytes from both substrains to kill YAC-1 cells. Furthermore, fresh splenocytes from both substrains were unable to lyse mammary tumor cells. We feel that these apparent inconsistencies are due to a major difference in the manner in which the effector cells were prepared. In experiments designed to study NK cell activity, preparation of effector cells included removal of nylon wool-adherent cells, while in work with LAK cells nylon wool separation was not employed. It is conceivable that exposure to nylon wool removes other cell types which could influence effector cell activity.

Mice of the C3H/OuJ substrain carry MMTV and have a high incidence of mammary adenocarcinomas, while C3Heb/FeJ mice are free of MMTV and seldom develop these tumors. The mechanism of MMTV-induced tumorigenesis is not completely understood. Most of the work in this area has focused upon the transforming effect of the retrovirus on mammary epithelial cells, and the model of insertional mutagenesis is compelling [26]. In the context of our work, it is interesting that a high level of viral antigen is expressed in lymphocytes [8]. Perhaps the virus plays a dual role in infected animals. On the one hand, it leads to the malignant transformation of mammary epithelial cells. On the other hand, it may weaken part of the immune surveillance mechanism of the host, possibly by inhibiting the production of IL-2, and thereby decreasing the ability to destroy neoplastic cells. The use of these two substrains of C3H mice provides a convenient model system for studying these effects and perhaps for gaining a better understanding of the role of NK and/or LAK cells in immunosurveillance against the initial development of spontaneous mammary tumors.

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