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Tumor-Associated Antigenic Differences Between the Primary and the Descendant Metastatic Tumor Cell Populations

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The existence of antigenic differences between cell populations in the local growth of the 3LL tumor (L-3LL) and its lung metastases (M-3LL) was studied. Normal C57BL/6 spleen cells sensitized in vitro for 5 days against L-3LL monolayers lysed preferentially L-3LL targets but not M-3LL tumor cell targets. Conversely, anti-M-3LL-sensitized lymphocytes killed M-3LL targets more efficiently than they killed L-3LL targets. Furthermore, spleen cells from mice bearing subcutaneous L-3LL tumors were significantly more cytotoxic to L-3LL targets than to M-3LL targets and vice versa. M-3LL cells were found also to be more resistant in vitro and in vivo to natural killer cells than were L-3LL tumor cells. M-3LL cells were more resistant than L-3LL cells to hybrid resistant mechanisms when they were inoculated into F_1 (C3Heb X C57BL/6) or F_1 (BALB/c X C57BL/6) mice. Anti-M-3LL lymphocytes generated both in vitro and in vivo, but not anti-L-3LL lymphocytes, admixed with L-3LL or M-3LL tumor cells and inoculated into footpads of syngeneic recipients suppressed the development of lung metastases. These results suggest that metastatic cells are indeed phenotypic variants of the local growing tumor cell populations. Presumably, these variants are selected for their capacity to home to and grow in the lungs, and for their resistance to specific immune effects initially evoked against the local tumor and to nonspecific natural killer cells. These data may prove to be of importance with respect to any rational approach to the problem of immunotherapy.

Key words: **hybrid resistance, NK cells, cytotoxic lymphocytes, tumor-associated antigens, primary and metastatic tumor cells, immunoselection**

The hope that immunologic methods may become extremely effective in the treatment of cancer patients is based on the notion that specific tumor-associated antigens are expressed on the tumor cell surface and that the host responds specifically against the progressing tumor.

Abbreviations used in this text: CL, cytotoxic lymphocytes; FCS, fetal calf serum; FUdR, fluorodeoxyuridine; ^{125}IUdR, ^{125}I-deoxyuridine; L-3LL, local growing 3LL tumor; M-3LL metastasis-derived tumor cells; NK, natural killer cells; SMS, syngeneic mouse serum; TBM, tumor-bearing mice.

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One may expect that these methods will be specially effective in the prevention of metastatic progression following surgical removal of the primary tumor mass. The search for appropriate immunotherapeutic methods has been intensified [1]. Modern immunotherapeutic approaches are based either on nonspecific stimulation of the immune system of the host or on the specific immunization of the organism against tumor cells originating in the surgically removed tumor. These immunization procedures are expected to stimulate the development of cytotoxic lymphocytes or antibodies which are capable of inhibiting tumor growth and destroying metastatic tumor cells. Another experimental approach to immunotherapy is based on the adoptive transfer to the diseased host of lymphocytes sensitized in vitro against tumor cells [1, 18].

All of the above-mentioned specific immunotherapeutic methods are based on the assumption that tumor cells originating in metastases are copies phenotypically identical to those tumor cells found in the primary tumor tissues and therefore that tumor cells originating in primary and metastatic tumors are expected to share identical tumor-associated cell surface antigens. Despite that attractive assumption, given the fact that most of the malignant tumors are heteroploid, one is forced to reexamine the validity of the above concept. Obviously the question is raised whether indeed metastatic cells are random representatives of the primary tumor cell population or whether they are selected out of a diverse population. The fact that tumor cells are characterized by impaired chromosome replication and segregation furnishes the basis for the generation of diversity among the primary tumor cell population. Such diversity may determine heterogeneity in genetic and phenotypic properties which in turn may furnish the basis for selective processes. In view of the above-mentioned immunotherapy goals, this question is of obvious importance. Variant cells with a higher probability of metastasis formation could be selected out [2]. Such an increased capacity for metastatic growth could be based a) on an increased probability of cell migration, b) on increased affinity for certain tissues, c) on preferential growth in the new tissue environment [2–5], and d) on resistance of the metastatic cells to the host's immune response directed against the primary tumor [6].

Indeed, recent studies have indicated that metastases may differ from the primary tumor cell population in a number of properties. Thus, differences in drug susceptibility [7], affinity to various organs [4, 5], chromosome number [8, 9], and some biochemical properties [10] have been observed.

The progression of metastasis may be a function of properties both of the host and of the neoplastic cells. Thus, it has been demonstrated that activation or suppression of the host's immune reactivity may result in decrease or increase of metastatic progression [11–13].

Metastatic spread may conceivably take place in organisms which respond immunologically against the primary tumor. The initial small populations of metastatic cells could escape immune destruction if their cell surface tumor antigens were different from those of the primary cell population. Immunoselection, as the basis for metastatic spread, would then predict that cells of tumor metastases differed antigenically from cells of the primary tumor. Furthermore, it has become clear in recent years that in addition to specific antigen-reactive lymphocytes, other lymphocytes manifesting cytotoxic effects against various types of tumors exist in the circulation and in various lymphoid organs [14–17]. These natural killer (NK) cells could then be effective against circulating tumor cells which could otherwise develop metastatic nodules. It seemed, therefore, of interest

to test whether cells derived from established metastatic nodules are different from tumor cells of the local primary growth in their susceptibility to the harmful effects of specific cytotoxic lymphoid cells as well as to NK cells. Such differences may be relevant to the control mechanisms involved in metastatic spread and growth.

We approached the problem of the possible diversity existing among primary and metastatic tumor cells by investigating the highly metastasizing Lewis lung carcinoma (3LL). Our investigation was aimed at testing a) antigenic and immunogenic differences existing between cells obtained from the local site of transplantation (L-3LL) and those cells obtained from metastases (M-3LL) which generally appear in the lungs of tumor-bearing mice; and b) the existence of differences in susceptibility of L-3LL and M-3LL tumor cells to the harmful activity of NK cells.

MATERIALS AND METHODS

Mice

Inbred male C57BL/6, C3H/eB, BALB/c mice (2–3 months old) and their F_1 hybrids F_1 (C3HeB × C57BL/6) or F_1 (BALB/c × C57BL/6) and C3H/eB/nu$^+$/nu$^+$ mice, were supplied by the Animal Breeding Center of the Weizmann Institute of Science. In some experiments C57BL/6 "B" mice were used. These were prepared as follows: Two-month-old C57BL mice were thymectomized; 1 month later they were irradiated (850 R) and reconstituted with 2×10^6 normal bone marrow cells. Two months following reconstitution, the presence of NK cells in the spleen was tested.

Tumors

Lewis lung carcinoma (3LL) was maintained by subcutaneous (SC) transfers of 1×10^6 tumor cells into syngeneic mice. The 3LL tumor produces metastases in the lungs following SC, intramuscular or intra-footpad (IFP) transplantation. Cell suspensions from solid tumors were prepared by treatment of minced tumor tissue with a solution of 0.3% trypsin (hog pancrease; Nutritional Biochemicals Corp., Cleveland, Ohio) [18]. The trypsin-treated cells were washed three times with phosphate buffered saline (PBS), pH 7.4, and resuspended in PBS for inoculation.

To obtain metastasis-derived tumor cells (M-3LL), 1×10^5 3LL tumor cells were inoculated in the hind footpads of C57BL/6 mice. Twenty-one days later mice were sacrificed and the developed pulmonary metastases were excised. In some experiments metastases were obtained from the lungs of mice 10–12 days following surgical removal of the local IFP growing tumor (8–10 mm in diameter). Tumor cell suspensions were prepared by trypsin treatment as above, and after PBS washing 1×10^6 tumor cells were inoculated SC into normal C57BL/6 mice. The cells derived from these tumors after a single SC transfer only were used as M-3LL. In parallel, 1×10^6 tumor cells obtained from the local 3LL SC transplanted tumor were inoculated SC to another group of mice and provided a source for the isolation of the local tumor growth (L-3LL).

Preparation of Sensitizing Monolayers

Subcutaneously growing M-3LL and L-3LL tumors were excised and suspensions of tumor cells were obtained by trypsin digestion The cells were washed three times with PBS and suspended in Waymouth's medium; then 5×10^6 tumor cells were seeded in 50-mm tissue culture plates (Falcon, 3002).

Preparation of Spleen Cells

Spleens from normal mice were removed aseptically, placed in cold PBS, and pressed through a fine stainless steel mesh. The cell suspensions obtained were washed twice with PBS, counted, and resuspended in RPMI-1640. The medium was supplemented with 1% fresh syngeneic mouse serum (SMS) [19].

Sensitization of Spleen Cells

The tumor cell monolayers were treated with 40 μg/ml of mitomycin C for 2 h, then washed twice with PBS and incubated for another 2 h with RPMI plus 1% syngeneic mouse serum. Then 30–40 $\times$ 10^6 splenic lymphocytes were suspended in 4 ml of RPMI-1640 and supplemented with 1% fresh SMS and 5 $\times$ 10^{-5} M 2-mercaptoethanol and seeded on top of the sensitizing monolayers. The lymphocytes were sensitized for 5 days at 37°C in a 10% CO_2 humidified air incubator.

On the 5th day, the sensitized lymphocytes were collected by gentle pipetting and separated from contaminating tumor cells by centrifugation in fetal calf serum. The collected cells were centrifuged and resuspended in undiluted fetal calf serum at a concentration of 10^7 cells/ml. This suspension was sedimented by centrifugation for 4 min at 6.5g and the cells in the supernatant were collected. These lymphocytes were washed three times with PBS and counted by trypan blue exclusion. By this process 15–30% of the initial number of lymphocytes cultured were usually recovered. Control normal lymphocytes were cultured for 5 days in the absence of a sensitizing monolayer.

Cytotoxic Activity of Cytotoxic Lymphocytes Generated In Vivo

The cytotoxic activity of splenic lymphocytes originating in tumor-bearing mice was tested following their precultivation for 2 days in vitro in the absence of sensitizing monolayers to "unmask" their cytotoxic activity [20].

In Vitro Assay of Natural Killer (NK) Cell Activity

The cytotoxicity exerted by normal spleen cells was tested against L-3LL and M-3LL target tumor cells by the same methods used in the assay of immune cytotoxic lymphocytes.

In Vitro Assay of Lymphocyte Cytotoxicity

We used the method described by Takasugi and Klein [21], as modified by More et al [22]. Briefly, 100,000 trypsinized tumor cells were pipetted into wells of microtiter plates (Nunclon-Delta flat-bottom Micro-Test tissue culture plates No. 1480). The cells were incubated for 24 h to permit adherence. The medium was then discarded and the target cells were washed three times with PBS. Following the last PBS wash, 10 control wells were trypsinized and the number of adherent cells was determined. Under these conditions, approximately 10% of the initial cell inoculum adhered to the bottom of the culture wells. Then the sensitized and control unsensitized lymphocytes were seeded (at various ratios relative to the final number of monolayer cells) into the wells. Cultures were incubated for 18 h at 37°C, the supernatants were removed, and each well received 50 μl Na_2 $^{51}CrO_4$ solution (Radiochemical Centre, Amersham, Bucks., UK), diluted in 0.4 M sucrose (5 μCi ^{51}Cr). The cultures were then incubated for 45 min at 37°C. The plates were stored on ice, washed, and then treated with 100 μl of 0.1 N NaOH for 10 min. The percentage of cytotoxicity was calculated according to the following formula:

$$\% \text{ lysis} = 1 - \frac{\text{cpm with test lymphocytes}}{\text{cpm with medium}} \times 100.$$

The percentage of cytotoxicity was expressed in negative values. Positive values represent increased uptake of isotope in test wells compared to control wells. Each percentage lysis value is based on six replicate samples.

In Vivo Assay for Tumor Growth

The immune or normal spleen cells were mixed with 2×10^4 M-3LL or L-3LL tumor cells at various ratios and the mixtures were grafted into the hind footpads of syngeneic mice. Control mice were grafted with tumor cells alone. The grafted mice were examined daily and the number of animals with visible palpable tumors were measured. Mice were sacrificed on day 21 following tumor inoculation; their lungs were removed and fixed in Bouin's solution. Visible metastases detected on the surface of the lungs were counted.

In some experiments the development of pulmonary metastases was assessed by determination of the degree of incorporation of ^{125}IUdR into the tested lungs; in these experiments we used mice in which the local IFP growing tumors, 8–10 mm in diameter, were surgically removed. Eleven days following tumor excision mice were inoculated with 25 μg/mouse of FUdR and 30 min later with 1 μCi of ^{125}IUdR [23]. Twenty-four hours following ^{125}IUdR injection the weight of lungs with metastases was determined and in parallel the degree of ^{125}IUdR incorporation by the malignant cells into the lungs was measured with a Packard Gamma Spectrometer. The significance of differences between various experimental groups was assessed using the Mann-Whitney U test [35].

RESULTS

In a previous study [19] we found that in vitro sensitization of lymphocytes against monolayers of syngeneic tumors carried out in culture media containing xenogeneic serum such as fetal calf serum (FCS) results in cytotoxic lymphocytes (CL) directed mainly against FCS determinants rather than against the actual cell surface tumor-associated antigens. We therefore studied the generation of CL when sensitization against syngeneic tumors was carried out in the presence of SMS. We demonstrated [19] that sensitization in SMS results in CL manifesting strict specificities against the tumor antigens.

Having found such strict antitumor specificities, we turned to test whether cells of the local growth of the 3LL tumor (L-3LL) possess antigenic specificities different from those of the metastatic population (M-3LL). We examined the specificity of the cytotoxic activity manifested by lymphocytes sensitized against monolayers of M-3LL compared to lymphocytes sensitized against L-3LL monolayers. The results described (Table I), summarizing three separate experiments, indicated that lymphocytes sensitized in fresh syngeneic mouse serum against monolayers of L-3LL lysed L-3LL targets significantly more than they lysed M-3LL cells. The activity against the L-3LL targets was very high, since even at ratios of lymphocytes to targets of 12.5:1 we got high levels of cytotoxicity. Conversely, lymphocytes sensitized against M-3LL cells lysed M-3LL targets significantly more than they lysed L-3LL targets. It appears, therefore, that each of these tumor populations is characterized, in addition to shared determinants, by specific cell surface antigens.

The specificity we obtained against metastatic cells in the experiments outlined above was manifested following sensitization in cell culture. The question arose whether the antigenic differences between M-3LL and L-3LL detected in vitro were not the result of in vitro cultivation of tumor cells. To answer this question, we investigated whether similar immunogenic differences between M-3LL and L-3LL are manifested in vivo, in the tumor-bearing mouse (TBM).

In earlier experiments, we found that freshly removed lymphoid cells from TBM are generally devoid of antitumor cytotoxic activity. Therefore, we incubated the spleen cells derived from TBM grafted with L-3LL or M-3LL cells for 48 h in the absence of sensitizing monolayer, to "unmask" their cytotoxic activity. The "unmasked" cytotoxic lymphocytes were then cross-tested for their cytotoxic activity against M-3LL and L-3LL tumor cells. We found (Table II) that splenic lymphocytes from mice bearing the L-3LL tumor lysed L-3LL targets significantly more than they lysed M-3LL targets. Conversely, cytotoxic lymphocytes from M-3LL TBM manifested an extent of lysis against M-3LL targets that was significantly higher than the lysis obtained against L-3LL targets.

These results indicate that the M-3LL and L-3LL grown in vivo express antigenic differences similar to those demonstrated by the in vitro analysis.

TABLE I. Specificity of Cytotoxic Activity of Syngeneic C57BL/6 Spleen Cells Sensitized In Vitro Against Local (L-3LL) or Metastatic Derived (M-3LL) Tumor Cells

		Net % lysis at lymphocyte-to-target-cell ratio of:				
Sensitizing tumor cells	Target tumor cells	Expt. 1 25:1	Expt. 1 12.5:1	Expt. 2 25:1	Expt. 2 12.5:1	Expt. 3 25:1
L-3LL	L-3LL	−54.6	−57.6	−49.3	−31.1	−45.7
L-3LL	M-3LL	−13.1	+ 4.4	− 5.2	− 0.4	− 3.7
M-3LL	M-3LL	−21.5	−31.4	−47.1	−46.5	−37.1
M-3LL	L-3LL	− 9.2	− 4.2	− 7.0	− 9.5	− 8.2

Normal C57BL/6 spleen cells were sensitized in vitro for 5 days on monolayers of L-3LL and M-3LL tumor cells. Negative values of cytotoxicity represent cell-mediated lysis by sensitized lymphocytes above the values obtained by unsensitized spleen cells.

TABLE II. Lytic Activity of Cytotoxic Lymphocytes Generated in M-3LL or L-3LL Tumor-Bearing Mice Against M-3LL and L-3LL Cells

		Expt. 1		Expt. 2	
Spleen cell donors[a]	Tumor target cells	Ratio of lymphocytes to targets	% net lysis	Ratio of lymphocytes to targets	% net lysis
L-3LL TBM	L-3LL	25 :1	−46	20:1	−30
		12.5:1	−25	10:1	− 6
M-3LL TBM	L-3LL	25 :1	− 8	20:1	−17
		12.5:1	− 1.4	10:1	+ 1
L-3LL TBM	M-3LL	25 :1	− 6	20:1	−12
		12.5:1	+ 7	10:1	+14
M-3LL TBM	M-3LL	25 :1	−27	20:1	−21
		12.5:1	− 1.2	10:1	−25

[a]Spleen cells were cultivated for 2 days in vitro without any sensitized monolayer.

The specificity we obtained against metastatic cells, using in vitro cytotoxicity assays, raised the question of whether anti-M-3LL cytotoxic lymphocytes generated in vitro or anti-M-3LL lymphocytes in M-3LL TBM are capable of suppressing the development of metastases in vivo. To test this we injected into the footpad either anti-L-3LL or anti-M-3LL in vitro-sensitized lymphocytes admixed with 2×10^4 L-3LL or M-3LL cells, at lymphocytes-to-tumor cell ratios of 25:1 or 12.5:1. We found no significant differences in growth of the local tumor at these cell ratios. However, when testing for the incidence of lung metastases at 21 days following cell inoculation, we found (Table III) that anti-L-3LL cytotoxic lymphocytes did not reduce the incidence of metastases produced by L-3LL cells (if anything, they increased the number of lung metastases). On the other hand, anti-M-3LL caused a significant reduction of metastases produced following the inoculation with L-3LL. A dramatic reduction was obtained at ratios of 25:1 lymphocytes to tumor cells. The effect of anti-L-3LL and anti-M-3LL cytotoxic lymphocytes on the production of metastases by M-3LL cells was also tested. Here again, anti-L-3LL cells did not reduce the incidence of lung metastases, whereas anti-M-3LL cytotoxic lymphocytes caused a reduction at lymphocyte-to-tumor-cell ratios of 25:1 and 12.5:1.

The "unmasked" cytotoxic lymphocytes obtained from spleens on the 14th day following SC inoculation with 1×10^6 L-3LL or M-3LL tumor cells were tested for their ability to suppress the development of metastases in vivo. "Unmasked" cytotoxic lymphocytes derived from either L-3LL or M-3LL tumor-bearing mice were mixed with 2×10^4 L-3LL or M-3LL cells at a ratio of 50:1 or 25:1 spleen cells to tumor cells and inoculated into the hind footpads of normal syngeneic recipients. When tumors reached 8–10 mm in diameter, the primary tumor was excised by amputation of the tumor-bearing leg. Twelve days following excision, mice were sacrificed and their lungs were examined for metastasis development (Table IV). Metastatic growth in the lungs was assayed by both weighing the lungs and measuring their ^{125}IUdR incorporation. In mice that had been inoculated IFP with L-3LL tumor cells and spleen cells of mice bearing L-3LL tumors (at ratios of 1:25 and 1:50), the metastatic growth in the lungs was accelerated, but the same spleen cells

TABLE III. Anti-M-3LL Lymphocyte Suppression of the Development of Lung Metastases

Sensitizing tumor cells	Transplanted tumor cells	No. of pulmonary metastases at ratios of sensitized lymphocytes to tumor cells equal to: 25:1	12.5:1
	Control, L-3LL cells alone	19.6	
None	L-3LL	21.7	27.3
L-3LL	L-3LL	28.6	35.2
M-3LL	L-3LL	3[a]	17.8
	Control, M-3LL cells alone	21.1	
None	M-3LL	42.4	N.T.
L-3LL	M-3LL	52[a]	42
M-3LL	M-3LL	7.7[a]	3.5[a]

Normal C57BL/6 syngeneic spleen cells were sensitized in vitro for 5 days on monolayers of primary local 3LL (L-3LL) and pulmonary metastasis-derived (M-3LL) carcinoma cells, in the presence of 1% syngeneic mouse serum. Sensitized cells were collected and transplanted at various ratios, together with either M-3LL, or L-3LL tumor cells, intra-footpad. The number of lung metastases was counted 21 days later. Each experimental group contained 10 mice.

[a]Differs significantly from control group according to Mann-Whitney U test ($P < 0.05$).

inoculated IFP admixed with M-3LL tumor cells failed to influence the development of metastases in the lungs of these mice (Table IV). Conversely, spleen cells originating in M-3LL-bearing mice manifested a slight inhibition of metastases when inoculated together with L-3LL tumor cells, but a high suppressing effect on metastases was observed when these "unmasked" cells were inoculated together with M-3LL tumor cells (Table IV).

Recent studies have suggested that nonspecific mechanisms mediated by cellular components of the immune system, namely naturally occurring killer cells (NK cells), may have a decisive function in controlling tumor development [14–17]. We therefore suggest that metastatic cells are not exempt from the defense mechanism and may be equally susceptible to injury by NK cells. Thus, it is expected that only a resistant fraction of tumor cells which survive in the blood and in various organs or tissues may be the main cellular source for initiation of distant metastases. Hence, it seemed of interest to investigate whether, indeed, cells of tumor metastases and those of the local tumor manifest differences with regard to susceptibility to the cytotoxic activity of NK cells. For that purpose experiments using different in vitro and in vivo approaches were performed to test the relative resistance of M-3LL and L-3LL tumor cells to injury by normal lymphoid cells.

The data described in Table V show that normal spleen cells are capable of killing 3LL tumor cells. The cytotoxic effect was exhibited by both freshly isolated normal spleen cells (Table V) and normal spleen cells cultured in vitro for 2 or 5 days (Tables VI,

TABLE IV. Suppression by Spleen Cells From Mice Bearing M-3LL Tumors, of Metastastic Growth in the Lungs of Tumor-Bearing Recipients

Treatment	Ratio between tumor and spleen cells	No. of mice	Weight of lungs, mg	cpm
L-3LL	–	10	438	13,635
L-3LL + Spleen N	1:25	10	424	12,433
	1:50	10	482	14,008
L-3LL + Spleen L-3LL	1:25	7	706[a]	21,403
	1:50	9	626[a]	25,672[a]
L-3LL + Spleen M-3LL	1:25	9	613	23,858
	1:50	8	355[a]	6,943[a]
M-3LL	–	10	671	25,338
M-3LL + Spleen N	1:25	9	703	29,189
	1:50	10	550[a]	13,467[a]
M-3LL + Spleen L-3LL	1:25	8	660	28,083
	1:50	9	584	17,060
M-3LL + Spleen M-3LL	1:25	10	547	19,759
	1:50	10	476[a]	9,892[a]

L-3LL or M-3LL tumor cells (1×10^6) were inoculated SC. On the 14th day of tumor growth spleen cells originating in these mice were cultivated in vitro for 2 days. These spleen cells were then collected and admixed at the above-described ratio with 2×10^4 L-3LL or 2×10^4 M-3LL tumor cells and inoculated IFP. The subsequently developed local tumors measuring 8–10 mm in diameter were removed, and 11 days following tumor excision mice were inoculated with 1 μCi of ^{125}IUdR, 24 h later mice were killed and both weight of lungs and radioactivity of lungs were determined.

[a]Differs significantly from control group, according to Mann-Whitney U test ($P < 0.05$).

VII). This cytotoxic effect of normal spleen cells was dose-dependent, eg, a clear increase in cytotoxicity was obtained by increasing the ratio of spleen to tumor cells in the microculture wells.

In parallel, experiments performed with M-3LL target cells presented in Tables V–VII clearly show that metastasis-derived tumor cells (M-3LL) were more resistant to the cytotoxic activity of normal spleen cells than L-3LL tumor cells.

Resistance of M-3LL cells to NK cells of freshly isolated spleen cells was similar to the resistance against spleen cells cultured for 2 or 5 days. In view of numerous works which have demonstrated that the cytotoxic activity of normal lymphoid cells is mainly mediated by NK cells [14–17], experiments were performed to characterize the nature of the effector cells involved in the cytotoxic injury to 3LL tumor cells. Results presented in Table VIII clearly demonstrate that spleen cells derived from either B mice or nude mice, or normal spleen cells treated by anti-Thy 1.2 serum and guinea pig complement, are still efficient in performing their cytotoxic activity against 3LL tumor cells. These characteristics are similar to those described for NK cells by other workers in the field [14, 24]. Thus, these results indicate that natural killer cells directed against the 3LL tumor are present in the spleen of immunologically intact C57BL/6 mice and are not eliminated during 2–5 days of in vitro culturing. The results indicate the existence of differences in the level of cytotoxic activity of NK cells between experiments (Tables V–VII). We attribute these differences to individual shifts in the spleen cell populations.

To test whether the differences in sensitivity of M-3LL and L-3LL tumor cells to the cytotoxic effects of NK cells observed in vitro have any in vivo significance, we tested the capacity of spleen cells derived from normal animals to inhibit the development of

TABLE V. Sensitivity of L-3LL and M-3LL Tumor Cells to the Cytotoxic Action of Fresh Unsensitized Normal Spleen Cells of C57BL/6 Mice

Expt. No.	Ratio between tumor and spleen cells	Target cells	
		L-3LL	M-3LL
1	1:200	−57.5	−19.0
	1:100	−35.6	−21.9
	1:50	−33.6	−14.9
	1:25	−28.8	−18.3
2	1:50	−40.0	−11.4
	1:25	−12.0	− 7.0
3	1:50	−17.0	−14.0
	1:25	− 6.0	+ 0.5
	1:12	+ 0.3	+19.0
4	1:50	−35.8	−16.0
	1:25	−21.9	− 5.2
5	1:200	−29.3	−15.4
	1:100	−23.3	−13.7

Fresh normal spleen cells of C57BL/6 mice were put into the wells on the monolayer of L-3LL or M-3LL tumor cells. Following 18 h survival, part of monolayers were labeled with ^{51}Cr. Negative values represent reduced uptake of isotope in test wells (as a result of cytotoxic activity) compared to control wells. Positive values represent increased uptake of isotope in test wells compared to control wells.

either M-3LL or L-3LL cells in intact syngeneic mice. M-3LL and L-3LL tumor cells were prepared and 2×10^4 cells of each tumor cell suspension were admixed with syngeneic normal spleen cells at a ratio of 1:25–1:200 tumor to spleen cells.

The mixture was inoculated into the hind footpads of syngeneic recipients. The development of tumors was assayed by recording the day of tumor appearance and tumor diameter, at various time intervals (Fig. 1a, b; Fig. 2a, b). The results (Fig. 1a, b) indicate an inhibition of L-3LL tumor growth when the ratio of inoculated tumor to spleen cells was 1:50 and 1:100. On the other hand, spleen cells enhanced the growth of M-3LL tumor cells at tumor to spleen cell ratios of 1:25 and 1:50 and inhibited the growth of M-3LL tumor cells only when the ratio of spleen to tumor cells was 1:100. Yet even at this ratio (1:100) normal spleen cells inhibited L-3LL cells significantly more than they inhibited M-3LL tumor cells. The percentage of inhibition of L-3LL tumors was 51–57%, whereas that of M-3LL tumors was only 25–26%.

In the subsequent experiments, ratios of 1:25–1:50 failed to influence the growth of L-3LL cells (Fig. 2a, b). Inhibition of L-3LL cells was observed only when the ratio between tumor cells and spleen cells reached 1:100–1:200. On the other hand, normal spleen cells mixed with M-3LL cells in a ratio of 1:25 accelerated tumor growth and had no significant influence on the development of M-3LL tumor cells when the ratios were 1:50 and 1:100. Only when the ratios reached 1:200 was a slight retardation of tumor growth obtained. At this high tumor to spleen cell ratio of 1:200, the growth inhibition of L-3LL tumor cells was 40–50% compared to 20–26% for M-3LL cells.

Since hybrid resistance and NK activity have common characteristics [25, 26] and since we aimed at testing the possible role of the MHC system in determining the relative resistance to L-3LL cells and their metastatic descendants, we studied a) in vitro NK activity against L-3LL and M-3LL cells and spleen cells from parental and F_1 origin and b) the

TABLE VI. Sensitivity of L-3LL and M-3LL Tumor Cells to the Cytotoxic Action of Normal Unsensitized Spleen Cells Cultured for 2 Days

Expt. No.	Ratio of tumor-to-spleen cells	Target cells	
		L-3LL	M-3LL
1	1:100	−51.5	−27.4
	1:50	−43.1	−14.0
	1:25	−28.8	−14.4
	1:12	−24.5	− 0.3
2	1:50	−31.7	−17.3
	1:25	−15.3	− 1.4
	1:12	− 7.7	− 1.7
3	1:40	−15.9	−10.2
	1:20	− 3.5	− 0.5
	1:10	− 5.9	− 1.8
4	1:40	−17.6	− 1.6
	1:20	− 5.2	+ 1.8
5	1:50	−35.9	−15.2
	1:25	−22.0	−16.0

Normal spleen cells of C57BL/6 mice were cultivated in RPMI medium + 1% syngeneic mouse serum for 2 days.

growth characteristics of L-3LL and M-3LL tumors in F_1 hosts. M-3LL and L-3LL tumor cells were subjected to lysis by freshly isolated spleen cells from syngeneic, allogeneic, and F_1 mice. The results (Table IX) indicate no H-2 restriction of the NK activity of normal spleen cells. Spleen cells derived from either syngeneic or allogeneic (ie, C3H/eB or BALB/c) and semiallogeneic [ie, F_1 (C3H/eB × C57BL/6) and F_1 (BALB/c × C57BL/6)] mice were equally efficient in their NK activity against 3LL tumor cells. Here again we found the relative resistance to NK cytotoxicity of M-3LL compared to L-3LL cells. Thus, irrespective of the haplotype of the donor, NK cells were more cytotoxic to L-3LL than to M-3LL cells.

We then tested whether the differences in susceptibility of M-3LL cells and L-3LL cells to the semiallogeneic NK cells observed in vitro would be reflected in vivo. Semiallogeneic recipients and syngeneic controls were inoculated in the hind footpads by either 1×10^5 M-3LL or L-3LL cells, and both tumor appearance and tumor diameter were recorded. Figures 3a, b and 4a, b indicate that there were no significant differences in the growth of either L-3LL or M-3LL tumor cells in syngeneic mice.

On the other hand, in both (BALB/c × C57BL/6) F_1 and (C3H/eB × C57BL/6) F_1 mice, a profound inhibition of L-3LL but not of M-3LL tumor development was observed. On the 15th day following inoculation of 1×10^5 M-3LL tumor cells, 100% of F_1 (BALB/c × C57BL/6) and 85% of F_1 (C3H/eB × C57BL/6) mice had established tumors. On the same day, only 55% of F_1 (BALB/c × C57BL/6) mice and 18% F_1 (C3HeB × C57BL/6) mice bore established L-3LL tumors. Even on the 26th day following the transplantation of L-3LL tumor cells, the percentage of F_1 (BALB/c × C57BL/6) and F_1 C3HeB × C57BL/6) hybrids bearing established L-3LL tumors was 75% and 58%, respectively. When compared with the growth of these tumors in syngeneic C57BL mice, M-3LL tumor growth was uninhibited in F_1 (C3HeB × C57BL/6) hybrids. In F_1 (BALB/c × C57BL/6 hybrids an accelerated growth of M-3LL tumors was observed (Figs. 3, 4).

TABLE VII. Sensitivity of L-3LL and M-3LL Tumor Cells to the Cytotoxic Action of Normal Unsensitized Spleen Cells Cultured for 5 Days

Expt. No.	Ratio of tumor-to-spleen cells	Target cells	
		L-3LL	M-3LL
1	1:50	−15.7	+17.8
	1:25	− 8.2	+ 3.4
	1:12	−17.6	+ 6.5
2	1:50	−21.7	−18.2
	1:25	−22.8	−13.8
	1:12	−20.0	+14.6
	1:6	− 5.6	+13.5
3	1:25	−15.2	+14.6
	1:12	−10.0	+13.5
4	1:25	−27.8	−15.0
	1:12	−14.2	+ 0.3
5	1:25	− 7.7	+ 3.4
	1:12	− 9.5	+10.2

Normal spleen cells of C57BL/6 mice were cultured in RPMI + 1% syngeneic mouse serum for 5 days.

DISCUSSION

In our previous study, we demonstrated that sensitization in culture of syngeneic lymphocytes against 3LL or B-16 tumors in the presence of syngeneic serum instead of the usually used xenogeneic serum, led to the generation of cytotoxic lymphocytes specific to these tumors [19]. In the present study, we applied the same approach to test whether antigenic differences exist between cell populations of the local growth of the 3LL tumor and its lung metastases. The results indicated that lung metastases which develop during the progression of an intra-footpad or SC inoculum of the tumor differ antigenically and immunogenically from the local tumor. Lymphocytes sensitized in culture against M-3LL lysed M-3LL targets more efficiently than they lysed L-3LL targets and vice versa. These differences are not the result of artifacts created by in vitro conditions, since spleen cells derived from L-3LL tumor-bearing mice, once "unmasked" in culture, lysed L-3LL cells, whereas M-3LL targets were relatively resistant. Conversely, spleen cells from M-3LL TBM lysed M-3LL significantly more than they affected L-3LL targets.

These results support the notion that the metastatic cells are indeed phenotypic variants of the local tumor growth. Thus, from a diverse tumor cell population strongly immunogenic migrating variants could be eliminated or suppressed during the early phases of tumor growth, by the immune reaction which they themselves evoke; clones which elicit a low, delayed, or different immune response could be selected out. Alternatively, because we use a serially transplantable tumor, one may assume that in addition to pre-existence of diversity, new antigenic variants may be generated de novo, during the progression of the local tumor. Fidler and Kripke [2, 27], studying the metastatic B-16 melanoma or the UV-induced fibrosarcoma, conducted experiments to test whether tumor cells with high metastatic potency preexist within the tumor cell population. They measured the incidence of lung metastases produced by intravenous inoculation of cell populations derived from different in vitro clones of the tumor cells. The results were that cell populations of different clones differed dramatically in their capacity to produce lung metastases. This suggests that in this tumor, cells with a higher metastatic potency pre-exist in the initial cell population. If this observation applies also to the 3LL tumor, it may account for the fact that anti-M-3LL CL manifested cytotoxicity against L-3LL

TABLE VIII. Cytotoxic Activity of T-Deprived Nonsensitized Spleen Cells Against 3LL Tumor Cells*

	% Cytotoxicity		
	Target-to-effector cell ratio		
Spleen cells	1:200	1:100	1:50
C57BL	−58	−33.2	−19.5
C57BL after anti-theta + C′ treatment	NT	−51.5	−38.3
B-mice[a] (C57BL)	−56	−40.1	NT
C3H	−43	−40.2	NT
C3H nude	−51	−38.8	NT

[a]B-mice: C57BL/6 mice 2 months old were thymectomized; 1 month later they were irradiated (850 R) and reconstituted with 2×10^6 syngeneic bone marrow cells from normal mice. Two months later, spleen cells of these mice were used in the cytotoxic test against 3LL tumor cells.
*C57BL, C3H (+/+), C3H (nu/nu) mice were 2 months old.

targets, although to a lower extent than against M-3LL (Tables I, II). The preexistence of tumor cells with a high metastatic potency is supported also by our observation that anti-M-3LL CL, when injected into mice admixed with L-3LL, caused a significant reduction in lung metastasis (Tables III, IV). Obviously, metastases produced by a growing subcutaneous tumor such as the tumors we investigated in the present study are determined by factors additional to those which determine the mere growth of metastatic tumor cells when these are injected intravenously. Thus, the very survival of tumor cells that detach from the local growth and migrate via the circulation might depend on their capacity to resist an immune reaction that the host directs against the growing local tumor, which, due to its large antigenic mass, can resist it. Tumor cells that a) can grow progessively in the lungs and b) are antigenically different from the cells of the local growth could be subjected to immune selection by the anti-L-3LL response that is evoked initially in the tumor-bearing animal.

The existence of antigenic differences between a local primary methylcholanthrene-induced tumor and its distant metastases was demonstrated by Sugarbaker and Cohen [29]. Using the method of cross analysis by immunization of mice against primary and metastatic tumors, these authors found that specific antigenic determinants are shared with primary tumor cells by some of the metastases and differ from others. In some cases, metastatic cells derived from the above-mentioned tumors lost their immunogenicity. Schirrmacher et al [30], using a methylcholanthrene-induced nonmetastasizing lymphoma Eb and its metastasizing variant ESb, have reported that these tumor cell lines

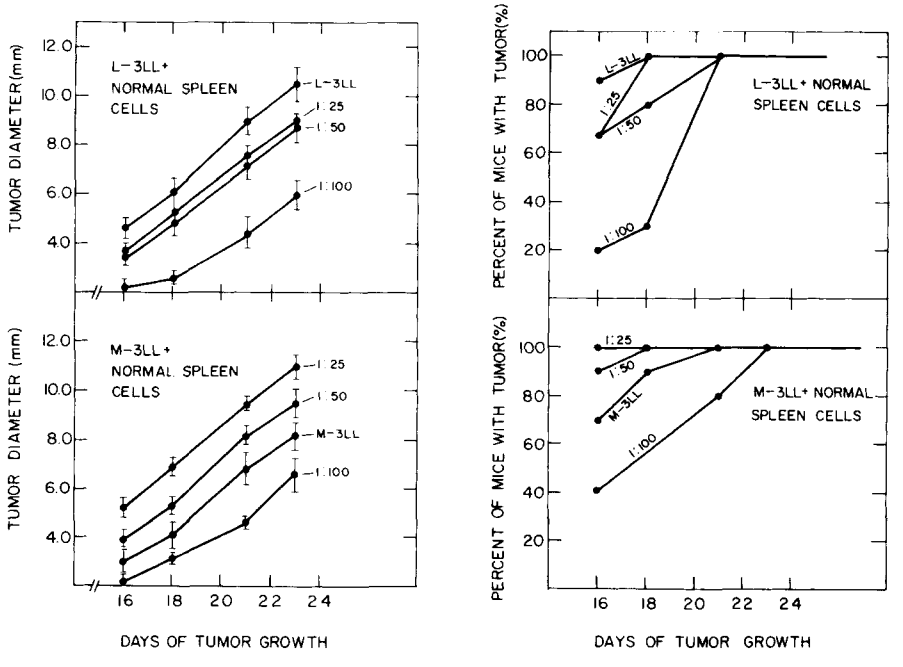

Fig. 1. In vivo sensitivity of L-3LL and M-3LL tumor cells to the inhibitory influence of fresh, unsensitized normal spleen cells of C57BL/6 mice. M-3LL or L-3LL tumor cells (2×10^4) were mixed with fresh normal syngeneic spleen cells at different ratios, as indicated in the figures. These mixtures were inoculated into the hind footpads of normal intact mice. At the indicated time intervals, both the percentage of mice with established tumors (b) and the mean tumor diameter (a) were recorded. Each group consisted of 10 mice.

carry distinct antigens at the responder cell level, the stimulator cell level, and the target cell level. These antigenic differences may be the result of an immunoselection of cells with distinct antigenic properties. This immunoselection may be the result of a specific immune response evoked against the local primary tumor.

Despite the suggestion outlined above our present results do not rule out an attractive assumption, namely, that NK cells themselves may act as an additional and independent selective force and may in fact themselves select antigenic variants manifesting higher resistance to specific and nonspecific cytotoxic effects. Therefore, in our present experiments we tested as well whether cells of the local growth, ie, L-3LL cells, manifest a susceptibility to NK activity which is different from that of M-3LL cells isolated from distant pulmonary metastases. We found that L-3LL cells are highly susceptible to lysis by splenic NK cells (Tables V–VII). In contrast, M-3LL tumor cells are relatively resistant to the cytotoxic activity of the same population of NK cells (Tables V–VII). The activity of NK cells is not directed against or affected by MHC or non-MHC alloantigens, because spleen cells from normal allogeneic or semiallogeneic F_1 hybrid mice are similar to syngeneic cells in their lytic effects and similar differences in susceptibility to allogeneic and semiallogeneic NK cells of L-3LL and M-3LL tumor cells are observed (Table IX). The cultivation of normal spleen cells in vitro for a period of 2 or 5 days did not abolish the cytotoxic function of NK cells (Tables VI, VII).

Experiments performed to characterize the nature of the effector cytotoxic cells present in the normal spleen cell population indicate (Table VIII) that these cells are not T cells and their characteristics are similar to those attributed in the literature to NK cells

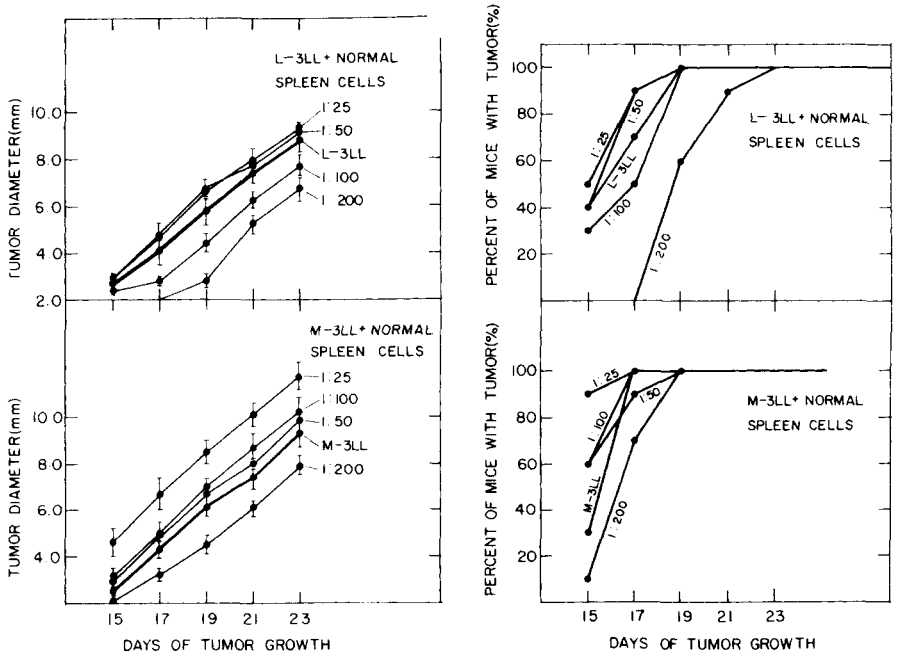

Fig. 2. In vivo sensitivity of L-3LL and M-3LL tumor cells to the inhibitory influence of fresh, unsensitized normal spleen cells of C57BL/6 mice. Conditions of experiment as described in Figure 1.

[17, 24]. Furthermore, we have performed experiments (Tables VI, VII) that strengthen the NK nature of the presently investigated effector cells because depletion of adherent cells failed to diminish the cytotoxic activity of normal spleen cells against the tumor cells. Yet our data so far do not rule out the possible involvement of another recently described population of natural cytotoxic cells (NC cells) which are non-T-cells and have been found to possess a few different characteristics than those attributed to NK cells [31, 32].

The differences in susceptibility of M-3LL and L-3LL tumor cells to the cytotoxic action of NK cells in vitro was also demonstrated in vivo; when spleen cells were admixed with tumor cells and inoculated into syngeneic recipients, the inhibition of L-3LL tumor growth was achieved by NK cells at relatively low doses. To inhibit the growth of M-3LL cells, higher doses of spleen cells were required. The inhibitory effect of M-3LL cells was lower than that obtained with L-3LL cells when admixed with spleen cells at similar ratios (Figs. 1, 2).

Although these results do not exclude the possible involvement of T cells and macrophages in the nonspecific defense against tumor progression in the diseased host, the data obtained in the present investigation indicate that the major nonspecific cytotoxic activity is performed by NK cells.

If indeed the mechanisms underlying NK cell activity and the phenomenon of hybrid resistance have a common basis, one would expect F_1 (BALB/c × C57BL/6) and F_1 (C3HeB × C57BL/6) mice to resist L-3LL tumor cells more than M-3LL growth. This, in fact, was the result we obtained (Figs. 3, 4). Our experiments thus clearly indicate that M-3LL and L-3LL tumor cells do differ in their susceptibility to the lytic activity of NK cells. The presently observed resistance of M-3LL tumor cells to NK cells might increase the probability of survival of metastatic cells in the circulation, thus providing an advantage for their spread and progression.

The possible role of NK cells in the elimination of tumor cells is supported by the results of Fidler and Nicolson [33], who demonstrated that following IV inoculation, radiolabeled B-16 melanoma cells that had settled in different organs were rapidly eliminated and only a very small fraction of the inoculated cells finally developed into metastatic foci. They found that 98.2% of the inoculated tumor cells that were arrested

TABLE IX. Cytotoxic Effect of Spleen NK Cells of Different Genotypes on L-3LL and M-3LL Tumor Target Cells

Strain of spleen donor mice	Expt. 1				Expt. 2			
	L-3LL		M-3LL		L-3LL		M-3LL	
	1:200	1:100	1:200	1:100	1:100	1:50	1:100	1:50
C57BL/6	−29.3	−23.3	−15.4	+13.7	−35.6	−33.6	−21.9	−14.9
BALB/c	−46.6	− 9.1	−25.8	− 9.3	−28.7	−37.2	−21.6	− 9.0
C3HeB	−28.8	−26.7	− 5.2	− 3.5	−39.2	−34.7	−20.4	−17.7
F_1 (BALB/c × C57BL/6)	−51.5	−18.4	+ 9.4	+31.9	−40.0	+ 4.3	−22.9	+ 5.9
F_1 (C3HeB × C57BL/6)	−46.2	−5.4	−11.6	+ 9.6	−53.6	−42.5	+ 0.4	−2.5

Fresh normal spleen cells obtained from immunologically intact mice of different genotypes were added to monolayers of L-3LL or M-3LL tumor cells for 18 h. The survival fraction of these monolayers was labeled with ^{51}Cr.

in the lungs were eliminated during the first day following inoculation and only 0.2% of transplanted cells developed visible metastases in the lungs [33]. Immunosuppressive procedures such as irradiation or thymectomy and irradiation did not abolish the elimination of the inoculated tumor cells. In fact, the elimination of tumor cells in the lungs and other organs 24 h following their injection was even greater in thymectomized and irradiated than in normal recipients. Although these authors have not considered the involvement of NK cells as part of the mechanism involved in the observed elimination of tumor cells, it is hard to believe that the elimination of tumor cells within a period of 24 h following IV inoculation is due to production of specific immune cytotoxic lymphocytes. On the other hand, it seems plausible that this rapid elimination of tumor cells is performed by preexisting NK cells. This assumption is supported by the fact that irradiation and thymectomy, which prevent the development of immune cytotoxic lymphocytes, failed to abolish activity of NK cells [17, 34].

The possible role of NK cells in tumor progression is further supported by a series of experiments in which Fidler and Nicolson [33] inoculated B-16 melanoma cells IV into allogeneic nude (nu/nu) and nu/+ heterozygote NIH Swiss mice. The allogeneic nu/+

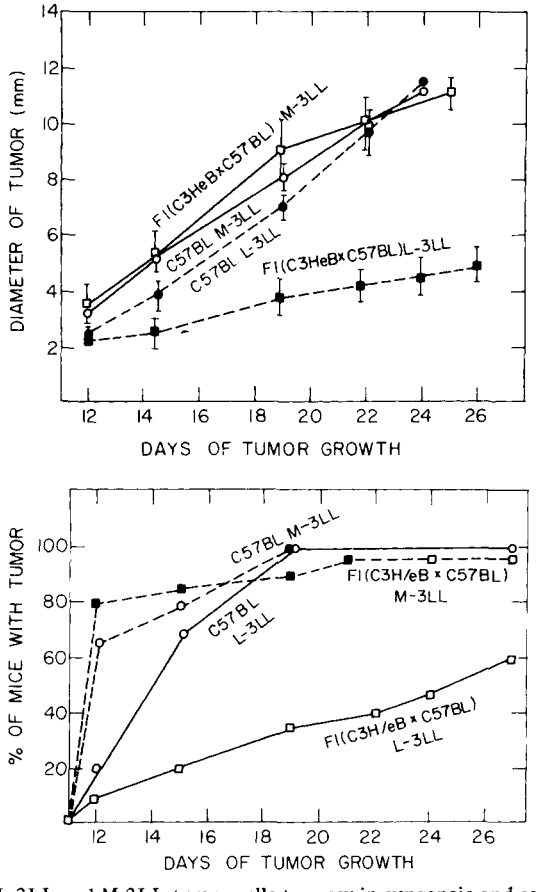

Fig. 3. Capacity of L-3LL and M-3LL tumor cells to grow in syngeneic and semiallogeneic hosts. M-3LL or L-3LL tumor cells (1×10^5) were inoculated into the hind footpads of normal intact F_1 or C57BL/6 control mice. At the indicated time intervals, percent of mice with established tumors (b) and mean tumor diameter (a) were recorded. Each group consisted of 40 mice.

mice killed inoculated tumor cells in a period of 24 h to the same extent as normal syngeneic mice. In the lungs of nude (nu/nu) mice, the percentage of surviving tumor cells was much lower than in normal allogeneic or syngeneic mice. These results are compatible with observations indicating that NK activity in nude mice is increased in comparison to their normal littermates [17, 34].

We therefore suggest that NK cells play an important role in determining metastatic spread and growth. The presently obtained results not only add an additional and new characteristic to the observed biologic differences between primary tumor cells and their metastatic progeny, but they might be of extreme importance and relevant to the mechanisms by which malignant tumors can disseminate their deviant progeny to form metastases in anatomically distant locations in spite of the existence of a protective physiologic mechanism provided by both NK cells and specific cytotoxic immunocytes. Furthermore, our presently described results, as well as those described by others [28], indicate that the development of malignant metastases is the result of a multifactorial process depending on many different phenotypic properties of the tumor cells themselves

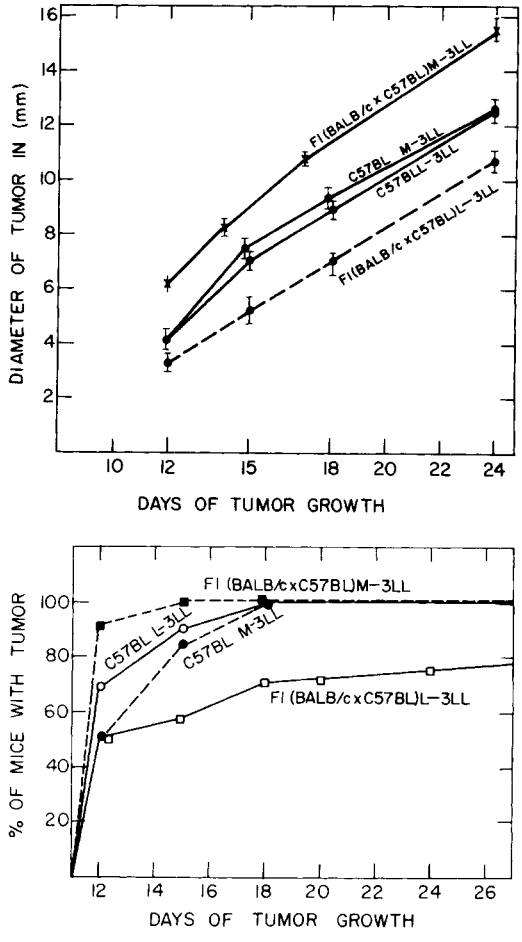

Fig. 4. Capacity of L-3LL and M-3LL tumor cells to grow in syngeneic and semiallogeneic hosts. Conditions of experiment as described in Figure 3.

as well as on a variety of both immunologic and nonimmunologic mechanisms in the diseased host. Whatever the relevance of the different antigenic properties of the metastatic cells to their biologic characteristics, such properties might be of extreme importance with respect to any future rational approach to the problem of immunotherapy of malignancies and may contribute to a better understanding of the unique physiologic characteristics of tumor metastases.

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