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Tumor Specificity, Serum Inhibition, and Influence of Regional Lymph Nodes on Cytotoxic Macrophages from Cultured Bone Marrow[1]

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SUMMARY

Prior investigations from this laboratory have demonstrated that, when bone marrow cells (BMC) from a tumor-bearing host are cultured, enhanced macrophage colony production occurs and macrophages from the colonies (CMA) are cytotoxic to tumor cells. The presently reported findings indicate that the cytotoxicity of CMA is highly specific, destroying only cells derived from the immunizing tumor. They support prior observations indicating that the cytotoxicity of the mature macrophage is derived from precursor stem cells and that no peripheral lymphocyte-antigen interaction with the macrophage is required for it to obtain that property. Information is also provided that indicates that cells from lymph nodes regional to a tumor (RLN) may affect stem cells from which CMA are derived. Not only is there decreased macrophage colony production when regional lymph nodes are absent, but also cytotoxicity of the resultant CMA is reduced. Observations indicating that transfer of regional lymph node cells from tumor-bearing mice to normal mice results in the production of cytotoxic CMA by BMC derived from the latter afford further support to this consideration. Findings also indicate that the cytotoxicity of CMA, like that of regional lymph node cells and BMC, is inhibited by serum from tumor-bearing animals and that the degree of inhibition increases with duration of tumor growth in the serum donor. Finally, it was observed that, with progressive tumor growth, BMC result in CMA with decreased cytotoxicity.

INTRODUCTION

Prior investigations from this laboratory have demonstrated a significant but transient increase of CFC[2] in bone marrow following implantation of a syngeneic mammary tumor in C3H mice. Those CFC gave rise to enhanced macrophage colony production when cultured in semisolid medium (2, 9). CMA were cytotoxic to cells from the immunizing tumor, and they continued to possess that characteristic for as long as a tumor was present in the animal from which the bone marrow was derived (11). Following tumor removal, cytotoxicity was rapidly lost. Additional studies have assessed the effects of prolonged cyclophosphamide and *Corynebacterium parvum* treatment on the production of CFC and on the cytotoxicity of macrophages comprising colonies produced by the CFC (10). Information indicating that the cytotoxic macrophage is an important effector cell in the inhibition of tumor growth was obtained by utilizing rifampicin, a semisynthetic antibiotic that interferes with macrophage function, but not with viability (7). All of the findings lend support to the contention that (*a*) the cytotoxic property of macrophages originates in ancestral stem cells or CFC in bone marrow and (*b*) receptor sites of the CFC (or stem cells) that respond to a stimulus for self-replication probably differ from sites that when activated produce progeny with cytotoxic properties.

This report presents results from studies carried out to obtain further information concerning the cytotoxic CMA. Those investigations have evaluated (*a*) the specificity of the cytotoxicity, (*b*) the role of RLN in the production of such cytotoxicity, and (*c*) the effect of duration of tumor growth on cellular cytotoxicity and on inhibition of the cytotoxicity by host serum.

MATERIALS AND METHODS

Mice. Inbred C3HeB/FeJ females, 8 to 12 weeks old, were housed in individual cages and fed laboratory chow and water *ad libitum*.

Tumors. A spontaneous mammary carcinoma arising in a C3H female and carried in female C3HeB mice was used. This tumor, designated as the C3H tumor, was used in all experiments. It was transferred by the injection of 2×10^5 viable tumor cells in 0.1 ml Medium 199 s.c. in the left hind leg at the ankle.

In experiments evaluating specificity of macrophage cytotoxicity, 2 different MC tumors were used, a mammary carcinoma (Tumor MCF) produced by gastric instillation of MC and a sarcoma resulting from s.c. inoculation of MC in C3HeB females.

Removal of RLN. The left popliteal and inguinal lymph nodes were removed under ether anesthesia either 7 days prior to tumor cell inoculation or on the day of inoculation.

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[2] The abbreviations used are: CFC, colony-forming cells; CMA, cultured macrophages; RLN, regional lymph nodes; MC, methylcholanthrene; RLNC, regional lymph node cells; BMC, bone marrow cells; MEM, minimal essential medium; LNC, lymph node cells.

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An equal number of control mice were subjected to ether anesthesia, skin incision, and suturing, but the lymph nodes were not touched. Mice were sacrificed from 1 to 28 days following tumor cell inoculation. The degree of cytotoxicity was not influenced by the time of node removal; consequently, the results have been combined for presentation.

Cells. The cells derived from the left popliteal and inguinal lymph nodes are designated as RLNC. Those cells obtained by flushing of the marrow cavity of femurs and assayed immediately for cytotoxicity are designated as BMC. Those cells harvested following *in vitro* culture of BMC for 7 days are referred to as CMA.

Bone Marrow Macrophage Assay. A modification of the method of Bradley and Metcalf (3) used by us in previous studies and described in detail elsewhere (2) was used. No colony-stimulating factor was utilized. All mice were killed by cervical dislocation, and 1 femur was promptly removed by sterile technique from each mouse. The marrow was removed by flushing from the cavity with Medium 1066 (Grand Island Biological Co., Grand Island, N. Y.). The cell suspension was counted and diluted to contain 1×10^6 cells/ml. Cell suspension (1.5 ml) was added to 13.5 ml of Medium 1066 containing 1.8% methyl cellulose and 15% horse serum. One ml of the resulting suspension containing 1×10^5 cells was plated in each of 10 tissue culture dishes (35 x 10 mm; Falcon Plastics Company, Oxnard, Calif.). Plates were incubated at 37° in a 10% CO_2 atmosphere with 100% humidity for 7 days. After that period of incubation, discrete colonies of 25 or more cells as well as small clusters of fewer cells were found. Identification marks on the plates were replaced by a code to ensure objectivity of the colony counts. Only groups of 25 or more cells arranged in a colony configuration were counted. The results are expressed as colonies/10^5 cells and colonies/femur. Statistical comparisons were made by the Student *t* test.

Production of Macrophages for Cytotoxicity Assay. To produce large numbers of macrophages, a colony-stimulating factor was added to the culture medium. BMC obtained as described above and diluted in Medium 1066 to contain 1×10^6 cells/ml were added to an equal volume of mouse L-cell-conditioned medium and 4 volumes of Medium 1066 with methyl cellulose and horse serum. Forty-ml aliquots were placed in 250-ml Falcon flasks and incubated for 7 days. At that time the cells harvested were, but for a rare exception, macrophages (4). The macrophages were isolated from the culture medium by 4 washings with 0.9% NaCl solution and resuspending in Earle's MEM with 30% fetal calf serum.

Preparation of RLN and BMC for Cytotoxicity Assay. Mice were killed by cervical dislocation. The RLN were removed, minced with scissors, and washed through an 80 mesh nylon screen with Earle's MEM with 30% fetal calf serum. The bone marrow was obtained as described above, an aliquot was resuspended in Earle's MEM, and the remainder was used for macrophage production. When RLNC and BMC were to be washed, the cell suspensions were divided into 2 aliquots. One was washed 6 times with Earle's MEM at room temperature, and the other (unwashed) was maintained at room temperature for the same period of time.

***In Vitro* Cytotoxicity Testing.** Cytotoxicity of macrophages, LNC, and BMC was assayed by the method previously described (8). The test was carried out in microtest plate wells covered by a monolayer of tumor cells. The cells to be assayed were added to a number of wells, and control cells or medium alone was placed in an equal number of wells. Cells of the same type that were used for negative controls were derived from non-tumor-bearing mice, whereas those serving as positive controls came from animals bearing a 14- to 28-day tumor. In all of these experiments, 5×10^4 cells were added to each well unless otherwise noted (Table 1). Plates, coded to eliminate bias in observation, were incubated for 48 hr, stained with crystal violet, and examined. A sufficient number of plates were used to provide 30 wells treated with each cell type in each experiment. The wells were scored on a scale of 0 to 5 according to the area free of tumor cells, with 0 indicating complete coverage and 5 indicating complete absence of tumor cells. The data are presented as the percentage of tumor cell destruction, which compares the amount of tumor remaining in the treated wells to that in the nontreated control wells. Data were analyzed by the Mann-Whitney *U* test.

Transfer of Cytotoxicity. A single-cell suspension was prepared in Hanks' balanced salt solution from RLN of mice bearing a 14-day tumor. It was diluted to contain 5×10^7 cells/ml. Each mouse received 5×10^6 cells via tail vein. LNC from non-tumor-bearing mice were prepared and injected in the same fashion. Mice were sacrificed at intervals of 3, 5, 7, 14, 21, and 28 days after transfer of LNC for determination of cytotoxicity of LNC, BMC, and CMA.

Sera for Inhibition Studies. Sera were obtained from mice after 2, 4, or 8 weeks of tumor growth or from normal non-tumor-bearing mice. They were bled individually from the retroorbital plexus, the blood was allowed to clot at room temperature, and the serum was removed and either used at once or stored at −70°. All sera were inactivated for 30 min at 56° and diluted 1:5 with phosphate-buffered saline (Dulbecco's phosphate-buffered saline without calcium or magnesium, pH 7.2, Grand Island Biological Company, Grand Island, N. Y.) just before use.

A sufficient volume of normal or tumor sera for a number of determinations was obtained by pooling an equal amount of serum from 6 to 12 mice. This pooled serum was dispensed in aliquots large enough for 1 day of use and stored frozen. No sera were refrozen and thawed.

In experiments designed to investigate serum inhibition of cytotoxicity, 3 mice were sacrificed after 14 days of tumor growth to provide bone marrow for culture. At that time, 3 mice with tumors of equal size were selected to be donors of RLNC and BMC 1 week later when the CMA were harvested. All 3 cell types were tested for inhibition of cytotoxicity by the 3 tumor sera. All testing of cell-sera combinations took place simultaneously on the same plates using the same target cell population. The experiment was repeated after 28 and 42 days of tumor growth.

For determination of whether the cytotoxicity of cells was influenced by the duration of tumor growth in animals from which they were derived, mice were given injections of tumor cells at intervals so that on the day of sacrifice there were mice with 14-, 28-, and 42-day tumors. One-half of

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each group provided bone marrow for culture, and the other mice with tumors of comparable size were used 1 week later for RLNC and BMC. All types of cells and both sera were applied to the same tumor plates, so that the conditions of the test were identical and comparisons could be made between all ages of cells. Only serum from animals with 56-day tumors was used.

For determination of cytotoxicity inhibition, the method described by Hellström *et al.* (15) was used. Target tumor cells in microcytotoxicity plate wells were incubated with each serum to be tested and an equal number of wells on each plate were treated with normal (non-tumor-bearing) mouse serum. After 45 to 60 min incubation of the plates at 37°, the sera were removed and the wells were washed once with phosphate-buffered saline. The cells to be assayed were added to a number of wells, and control cells or medium alone was placed in an equal number of wells. Cells of the same type derived from the non-tumor-bearing control mice were used as negative controls.

RESULTS

Influence of Duration of Tumor Growth on Cellular Cytotoxicity and Serum Inhibition

Inhibition of cytotoxicity of each of the 3 cell types (CMA, BMC, and RLNC) was found to increase in direct relationship to the length of tumor growth in the mice from which the sera were derived (Chart 1). This was consistently observed when the various sera were used with cytotoxic cells that were obtained from animals with tumors that grew for different lengths of time. In each experiment, the reduction of cytotoxicity by 14- or 28-day serum was proportionally less when CMA were tested than when either BMC or RLNC were used. When serum from animals bearing tumors for 56 days was used, a relatively uniform inhibition of cytotoxicity by the 3 cell types existed.

The cytotoxic inhibition of each cell type was dependent not only on the duration of tumor growth in animals from which serum was derived but also on the duration of tumor growth in animals from which the cells to be tested came. The cytotoxicity of either CMA, RLNC, or BMC was significantly decreased ($p < 0.001$) by an additional 4 weeks of tumor growth in animals from which the cells were derived. This was observed when either normal serum or serum from 56-day tumor-bearing animals was used (Chart 2).

When normal serum was used with RLNC obtained from animals with 7-week tumors, there was a significantly greater reduction in cytotoxicity than when RLNC came from animals with 3-week-old tumors. When tumor serum was used, there was an even greater decrease in cytotoxicity in cells from animals with the older tumors.

Findings with BMC and CMA were similar to those with RLNC. With normal serum, a significant reduction in cytotoxicity was associated with the cells derived from mice having an additional month of tumor growth. With tumor serum the reduction was even greater when cells came from animals with older tumors.

To determine whether the decreased cytotoxicity of RLNC and BMC, after a long period of tumor growth, was due to the presence of an extrinsic factor on the cell surface, these cells were repetitively washed prior to being placed on cytotoxicity plates. Such washing increased the cytotoxicity of all cell suspensions (Table 1). There was no increase in the cytotoxicity of macrophages from cultured BMC that had been washed prior to their culture.

Specificity of Macrophage Cytotoxicity

When cytotoxicity was assayed by exposing either C3H or MCF tumor cells to CMA from animals bearing a tumor of the same cell type, a high degree of cytotoxicity (60 to 70%) was obtained (Table 2). When the immunizing tumor was different from that which provided the target cell, a degree of cytotoxicity was observed that was only slightly greater

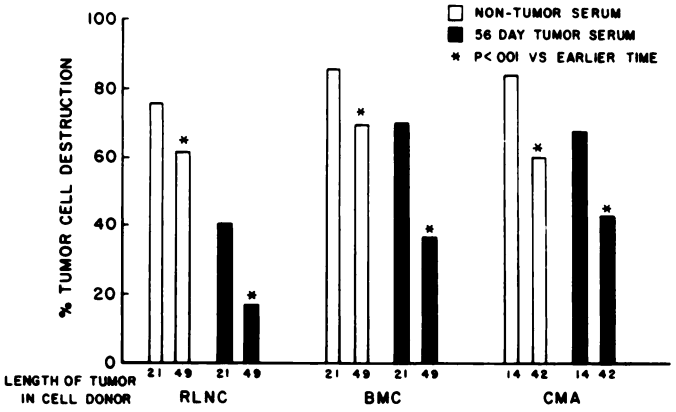

Chart 2. Effect of increasing time of tumor growth of cellular cytotoxicity and response to serum inhibition.

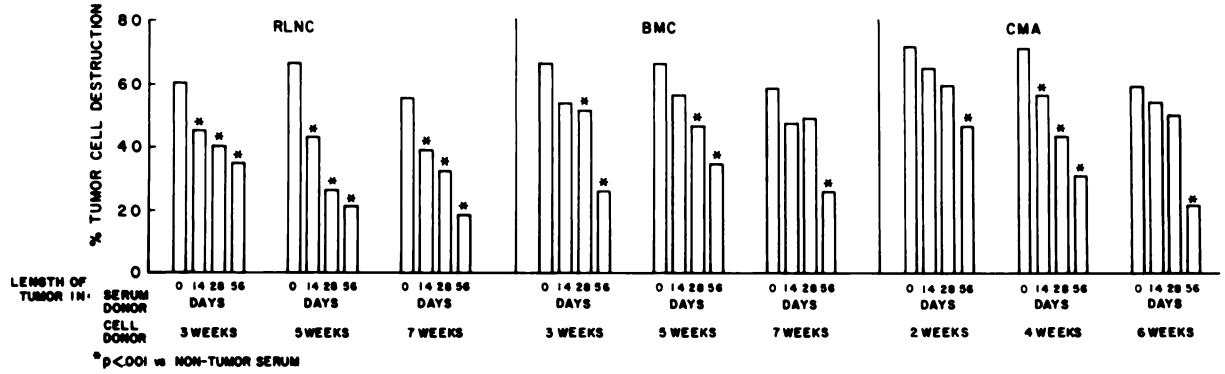

Chart 1. Effect of serum from tumor-bearing mice on cytotoxicity of RLNC, BMC, and CMA.

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than that produced by macrophages from normal mice. When the chemically induced tumors were used for immunization, the macrophages from those animals were more cytotoxic against the C3H tumor than *vice versa*. Similar findings were obtained when the cytotoxic cells were from bone marrow or RLN.

Role of RLN in the Production and Cytotoxicity of CMA

Effect of Absence of RLN. At 3 days of tumor growth, the macrophage colony production by CFC was significantly decreased when RLN had been removed either 7 days before or just prior to tumor cell inoculation. This was evident (Table 3) when results were presented either as colonies/10^5 BMC or as colonies/femur. Similar observations were obtained when the BMC were cultured after 7 or 14 days of tumor growth.

At all times investigated (from 1 to 28 days of tumor growth), cytotoxicity of BMC and CMA was greater when RLN were present in animals with tumors (Table 4).

Transfer of RLNC to Non-Tumor-bearing Mice. The transfer of RLNC to normal animals resulted in the produc-

Table 1

Effect of repeated washing on cytotoxic property of effector cells

Cytotoxic cell	Days of tumor growth of cell donor	No. of cells/well × 10^{-3}	Unwashed cells	Washed cells
RLNC	40	25	32.2[a]	57.5
	40	12.5	21.4	52.2
BMC	40	25	19.9	49.3
	40	12.5	12.3	41.1
CMA	21	12.5	24.6	24.1
	50	12.5	17.3	13.8
	21	12.5	36.3	27.5

[a] Percentage of tumor cell destruction.

Table 4

Effect of the absence of RLN on development of cytotoxicity in BMC and CMA during tumor growth

Day of tumor growth	Index of tumor destruction: BMC, Lymph nodes present	BMC, Lymph nodes absent	CMA, Lymph nodes present	CMA, Lymph nodes absent
1	3.9[a]	2.0	2.6	1.2
2	5.9	3.2	6.7	3.2
3	17.1	11.9	12.9	7.1
5	25.1	14.1	21.4	15.0
7	34.1	23.9	41.5	32.5
14	67.4	62.5	68.8	64.1
28	73.3	62.5	69.3	64.2

[a] Percentage of tumor cell destruction.

Table 2

Specificity of RLNC, BMC, and CMA cytotoxicity

Immunizing tumor	Target tumor cell	Mice with 14-day tumor: RLNC[a]	BMC	CMA	Mice with 28-day tumor: RLNC	BMC	CMA
None	C3H	3.1[b]	3.1	2.2	1.0	1.0	1.0
None	MCF	1.2	1.2	0.6	1.0	1.0	1.0
C3H	C3H	64.7	70.8	72.4	67.4	72.9	74.1
C3H	MCF	1.6	2.8	3.2	2.1	3.5	4.1
MCF	C3H	9.2	7.7	8.1	5.6	10.2	13.7
MCF	MCF	61.6	63.1	60.8	67.8	71.9	69.2
MC	C3H	6.7	5.3	8.1	1.3	4.5	4.1
MC	MCF	6.8	6.6	7.3	5.6	8.3	10.3

[a] Effector cells.
[b] Percentage of tumor cell destruction.

Table 3

Effect of absence of RLN on macrophage colony production during tumor growth

Days of tumor growth	Lymph nodes removed	Colonies/10^5 BMC[a]: 1. Lymph nodes present	2. Lymph nodes absent	Colonies × 10^{-3}/femur[a]: 3. Lymph nodes present	4. Lymph nodes absent
1	A[b]	44.7 ± 2.09	45.9 ± 2.78	5.8 ± 0.35	6.0 ± 0.36
	B	60.0 ± 2.31	62.0 ± 2.31	6.9 ± 0.21	6.9 ± 0.36
3	A	54.6 ± 1.56	43.6 ± 1.64[c]	5.1 ± 0.15	4.2 ± 0.15[c]
	B	108.8 ± 9.89	73.1 ± 3.10[c]	10.4 ± 0.45	6.2 ± 0.32[c]
7	A	57.0 ± 1.71	44.8 ± 1.41[c]	5.0 ± 0.17	4.0 ± 0.11[c]
	B	99.4 ± 2.92	83.3 ± 2.92[c]	9.9 ± 0.47	8.6 ± 0.30
14	A	56.8 ± 1.91	41.0 ± 1.62[c]	4.8 ± 0.28	4.0 ± 0.17
	B	90.0 ± 2.70	72.6 ± 3.01[c]	9.2 ± 0.52	7.3 ± 0.67[c]

[a] Mean ± S. E.; 10 plates/experiment.
[b] *A*, 7 days prior to tumor cell inoculation; *B*, day of tumor cell inoculation.
[c] $p < 0.01$ for 1 *versus* 2 and 3 *versus* 4.

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tion of cytotoxic CMA, which was maximum at 7 days. While the degree of cytotoxicity subsequently declined for at least as long as 28 days, that attribute of CMA remained greater than did similar cells from animals receiving LNC from non-tumor-bearing animals (Chart 3). BMC and LNC displayed a similar development of cytotoxicity following transfer of RLNC from tumor-bearing mice, except in that the maximum cytotoxicity of LNC was observed at 14 days.

DISCUSSION

Lymphoid cells from animals with growing tumors are cytotoxic *in vitro* to neoplastic cells of the same line. Sera from such hosts interfere with cytoxicity of those lymphoid cells (12–14). There is general agreement that this interference appears early and increases with progressive growth of tumor (1). The present findings are in agreement. While some investigators have found no alteration in the cytotoxic properties of the lymphoid cells during the growth of a tumor (1), others have reported a decrease (5, 16). This reduction in cytotoxicity may be a reflection of changes in the population of cells being tested. There may be either a larger proportion of noncytotoxic cells or a reduction in specific cell cytotoxicity. The present findings indicate that with progressive tumor growth there is a reduction in cytotoxicity, probably associated with accumulation of soluble serum-inhibitory factors on the cell surface which is readily removed by washing, with no intrinsic alteration of the cytotoxic cell. On the other hand the reduction in cytotoxicity of CMA seems to be a quality that is intrinsic to the cell and that is probably related to alterations in its antecedent stem cell, since intensive washing of BMC prior to culture failed to increase the cytotoxicity of the resulting macrophages.

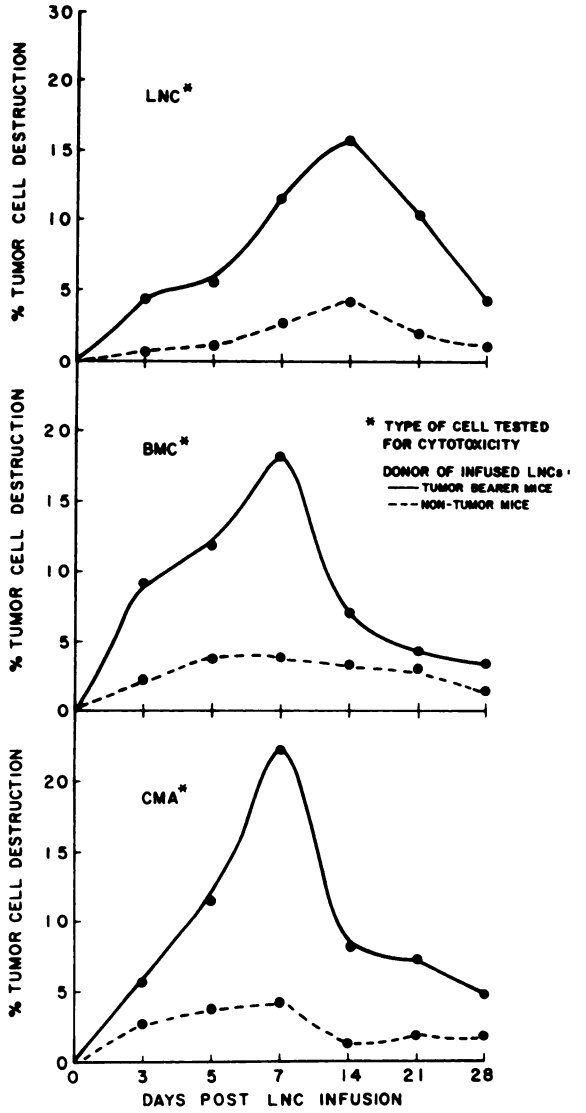

Chart 3. Development of cellular cytotoxicity after transfer of RLNC from tumor-bearing to normal mice.

The findings demonstrate for the 1st time that the cytotoxicity of CMA is also inhibited by serum from animals bearing tumors and that the degree of inhibition of those cells increases with the duration of tumor growth in the serum donor. If *in vitro* cytotoxicity is a correlate of cell-mediated tumor cell destruction *in vivo*, and if the macrophage is a cell of primacy in such destruction, then the findings suggest that the adverse effects of the serum factor(s) may be more pervasive than merely its interference with lymphocyte cytotoxicity.

It has been demonstrated that mature macrophages from a tumor-bearing host (peritoneal, etc.) become specifically or nonspecifically cytotoxic to neoplastic cells (6). The specificity of the cytotoxicity of the CMA required evaluation. The present findings indicate that this attribute of those cells is indeed a specific one and that the specificity is acquired from the macrophage precursor stem cells in animals bearing a tumor. In that regard, the findings may be considered in the light of those of Evans and Alexander (6), and associates. They have indicated that "arming" is a function of the mature macrophage that is mediated through hyperimmune spleen cells or the supernatant of immune lymphocytes cultures with specific antigen. These cells then become specifically cytotoxic. Our findings suggest that a mature macrophage in a tumor-bearing host could already possess specific cytotoxic properties and that no peripheral lymphocyte-antigen interaction with it would be required for its arming since that function may have already occurred prior to maturation of that cell. Thus, while there is no disagreement with the findings of Evans and Alexander and associates regarding the arming of mature macrophages *in vitro*, such a mechanism may be less important in the tumor-bearing host.

Findings regarding the role of the RLN and the development of cytotoxicity in CMA are of interest. They suggest that, while the presence of RLN is not imperative to the development of BMC or CMA cytotoxicity, the degree of cytotoxicity was slightly reduced by their absence. That effect was more evident early in tumor growth suggesting that RLNC may play a role in the "priming" of the stem cells from which the CMA are derived. The observations obtained, indicating that transfer of RLNC to non-tumor-bearing mice resulted in the production of cytotoxic CMA, supports this consideration. Further evidence indicating the existence of a relationship between nodes regional to a

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tumor and bone marrow is evidenced by the decrease in macrophage colony production when RLN were absent. The precise mechanism whereby stem cells become specifically cytotoxic remains to be elucidated.

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