The Effect of a Growing Tumor and Its Removal on the Cytotoxicity of Macrophages from Cultured Bone Marrow Cells

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SUMMARY

Previous investigations by us have demonstrated that there is a significant but transient (4 to 14 days) increase of colony-forming cells (CFC) in bone marrow following implantation of a syngeneic mammary tumor in C3H mice. Those CFC gave rise to enhanced macrophage colony production when cultivated in semisolid medium. The present studies have demonstrated a transient increase in macrophage CFC during the growth of a syngeneic tumor (2).

The present investigations were carried out to determine whether the increased macrophage production by a host in response to a tumor resulted in newly formed cells with properties that could be associated with tumor cell destruction. Cells from lymph nodes regional to growing C3H mammary tumors (19) are cytotoxic, and the cytotoxicity decreases rapidly following removal of the tumor (13, 15, 19). It was considered pertinent to ascertain whether macrophages comprising the colonies produced by cultured bone marrow cells displayed a similar pattern of cytotoxicity.

MATERIALS AND METHODS

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

**Tumors.** A spontaneous mammary carcinoma arising in a C3H female and carried in female C3HeB/FeJ mice was used. This tumor is designated as the C3H tumor. It was transferred by s.c. trocar inoculation of a 1- to 2-mm fragment of tumor into the left hind leg distal to the popliteal nodes. Tumors 5 mm in diameter developing by 14 days were removed by amputation of limbs at the knee so as to leave the regional lymph nodes intact.

**Bone Marrow Macrophage Culture.** A modification of the method of Bradley and Metcalf was used (4). All animals were killed by cervical dislocation. They were immersed in antiseptic solution and 1 femur was promptly removed from each by sterile technique. The marrow obtained from femurs of 3 mice in each group was pooled. Cells were removed from the femurs by drawing Medium 1066 through the marrow cavity. The cell suspension was counted, and 1 × 10^6 cells/ml. The diluted cell suspension was added to an equal volume of mouse L-cell-conditioned medium and 4 volumes of Medium 1066 containing 1.8% methylcellulose and 15% horse serum. The material was removed for 15 min using a magnetic stirring bar, and 40-ml aliquots were placed in 250-ml Falcon flasks. After 7 days of incubation in a 10% CO₂-100% humidity atmosphere, the semisolid culture medium contained colonies, i.e., groups of macrophages. The medium was removed by diluting it with 0.9% NaCl solution and centrifuging at 450 × g for 25 min at room temperature. After 4 washes to remove particles of methylcellulose, the

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2 The abbreviation used is: CFC, colony-forming cells.
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isolated macrophages were suspended in Earles minimal essential medium with 30% fetal calf serum. The final suspension of macrophages contained 95 to 100% viable cells by the trypan blue exclusion test.

In Vitro Cytotoxicity Testing. The cytotoxicity of the macrophages was assayed by the method previously described for lymph node cells (13). The test was carried out in microtest-plate wells covered by a monolayer of tumor cells. The macrophages to be assayed were added to a number of wells, and, as controls, macrophages from nontumorous mice, regional lymph nodes from tumor-bearing mice, or medium alone was placed in an equal number of wells. In all of these experiments 5 x 10^4 cells were added to each well. Plates that were coded so as to eliminate bias in observation were incubated for 48 hr, stained with crystal violet, and examined. The wells were scored on a scale of 0 to 5 according to the area free of tumor cells: zero, complete coverage and 5 indicating complete absence of tumor cells; Grade 1, tumor cell destruction of 1 to 25%; Grade 2, 25 to 50%; Grade 3, 50 to 75%; and Grade 4, 75 to just less than 100%. The data are presented both as the index of tumor cell destruction representing the mean ± S.D. of at least 30 observations and as the percentage of tumor cell destruction. The latter compares the amount of tumor remaining in treated wells to that in nontreated control wells. Data were analyzed by the Student t test for significance at a level of 0.001. In all experiments the degree of tumor cell destruction by a given cell type was uniform, there being little variation from well to well.

Experimental Design. In experiments with tumor-bearing mice (concomitant immune), bone marrow cells were obtained from animals after 3, 5, 7, 14, 21, 28, 31, 35, 42, or 56 days of tumor growth. In sinicomitant immune mice, regional lymph nodes from tumor-bearing mice, or medium alone was placed in an equal number of wells. In all these experiments 5 x 10^4 cells were added to each well. Plates that were coded so as to eliminate bias in observation were incubated for 48 hr, stained with crystal violet, and examined. The wells were scored on a scale of 0 to 5 according to the area free of tumor cells: zero, complete coverage and 5 indicating complete absence of tumor cells; Grade 1, tumor cell destruction of 1 to 25%; Grade 2, 25 to 50%; Grade 3, 50 to 75%; and Grade 4, 75 to just less than 100%. The data are presented both as the index of tumor cell destruction representing the mean ± S.D. of at least 30 observations and as the percentage of tumor cell destruction. The latter compares the amount of tumor remaining in treated wells to that in nontreated control wells. Data were analyzed by the Student t test for significance at a level of 0.001. In all experiments the degree of tumor cell destruction by a given cell type was uniform, there being little variation from well to well.

**RESULTS**

In all experiments (Table 1) macrophages from cultured bone marrow cells of mice with 3- to 56-day-old tumors were significantly more cytotoxic to C3H tumor cells than were such cells obtained from marrow of nontumorous animals. Within 7 days after removal of a tumor, a significant reduction in cytotoxicity of macrophages was evident (Table 2). Macrophages from marrow cells harvested 21 days after tumor amputation were found no longer to possess cytotoxic properties.

**DISCUSSION**

These investigations have, for the 1st time to our knowledge, clearly demonstrated that macrophages produced by culturing bone marrow from tumor-bearing animals are cytotoxic to cells from the immunizing tumor. Such cytotoxic properties persisted as long as a tumor was present. It had been previously demonstrated by us (2) that increased numbers of CFC occurred only during the initial phase of tumor growth. Although macrophage colony production was not determined in this series of experiments, during the same time that these studies were carried out repeated assays of CFC in bone marrow of tumor-bearing animals during the 4th to 8th week of tumor growth were done in other investigations. They failed to demonstrate greater numbers of CFC than found in normal mice. If the present findings regarding macrophage cytotoxicity are related to those obtained by us regarding macrophage colony production, several hypotheses may be formulated. Since the degree of cytotoxicity of macrophages remained relatively constant during growth of a tumor whereas numbers of CFC changed, it is considered that factors responsible for regulating the population of CFC do not selectively stimulate or inhibit those CFC responsible for production of cytotoxic macrophages. Several possibilities may be considered regarding the genesis of macrophage cytotoxicity. Since macrophages are descendants of CFC, cytotoxicity may have had its origin in the CFC or even in stem cells which are precursors of the CFC. How changes might occur in CFC to account for their giving rise to the cytotoxic macrophage is conjectural. Whether all or a precommitted subpopulation of CFC is directly responsive to antigens transmitted to bone marrow or whether the cells are indirectly affected by sensitized lymphocytes from regional lymph nodes, spleen, and/or thymus remains to be determined.

Alternatively, it may be considered that the cytotoxic property of macrophages had been acquired during their presence in culture. Since the entire bone marrow population, of which CFC make up only a very small proportion, has been placed into the culture medium, the possibility that cells other than the macrophages either are responsi-
ble for the cytotoxicity or are capable of "arming" macrophages in a fashion analogous to mechanisms described by Evans and Alexander (10) might be postulated. That cells other than macrophages are responsible for the cytotoxicity observed may readily be discounted since it has been established that cells of the myeloid type, i.e., granulocytes and/or macrophages, are the exclusive inhabitants of the colonies produced by the particular method of culture used (5).

That cytotoxicity is the result of interaction between the macrophage and another cell type, e.g., a sensitized lymphocyte, in the semisolid culture medium also seems unlikely, for cells when initially plated are so diffusely dispersed in the culture medium that extensive lymphocyte migration through the medium would be necessary for a direct cell interaction to take place even should such cells persist in culture, a finding that has not been observed. The possibility that a factor is elaborated by the non-CFC which diffused through the culture medium or that antigen transmitted into the culture along with the marrow could affect the maturing macrophages has been considered and, although not entirely disproven, remains an unlikely possibility.

Whatever proves to be the ultimate explanation for the observed findings, they are of importance in that they have demonstrated that, at least in the model used, macrophages derived from cultured bone marrow cells of tumor-bearing mice possess specific cytotoxicity that seems to originate from the ancestral CFC or stem cell.

The reason for loss of cytotoxicity by the macrophages following tumor removal is uncertain. It has also been demonstrated by us that regional lymph node cells lose cytotoxicity following tumor removal (7). Perhaps loss of tumor antigen may be implicated, either through its direct effect on CFC or indirectly through the lymph node cells. Investigations to determine the chronology of the development and loss of cytotoxicity in regional lymph node and in bone marrow cells, cultured and uncultured, are in progress further to elucidate the mechanism involved.

Since the cytotoxicity of macrophages persists at a time when there is no longer evidence that CFC production is being stimulated, it is possible that receptor sites of cells which respond to a stimulus for CFC formation are different from sites which when activated result in cytotoxic properties of their progeny. Although all stem cells (or CFC) probably possess the capability of being stimulated to form increased numbers of CFC, the capacity for transmitting cytotoxicity may not be so universal and may be related to a specific stimulus. The gradual but complete loss of cytotoxicity following tumor removal tends to support the concept that the physical presence of antigen is necessary for maintenance of cytotoxicity, and the possibility is suggested that cytotoxicity is not passed on by replication of stem cells.

It had been suggested by us (19) that increased bone marrow macrophage colony production might be used as a parameter for determining effectiveness of an optimum antitumor therapeutic regimen. No consideration was given at that time to the importance of the cytotoxicity of such macrophages. The present findings suggest not only that quantitative determinations of macrophage production are important but also that specific properties, i.e., cytotoxicity, of such cells are relevant. Although increased macrophage production may be advantageous for the performance of a variety of functions such as augmenting antigen processing, clearing antigen-antibody complexes, and phagocytizing dead tumor cells, it is the cytotoxic macrophage that, by directly destroying tumor cells, may be of prime importance in the host effector response to a tumor.

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