Cooperation of Immune Lymphoid and Reticuloendothelial Cells during Listeria monocytogenes-mediated Tumor Immunity

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SUMMARY

Listeria monocytogenes (LM) in admixture with cells from a murine, chemically induced tumor retarded local tumor development in the syngeneic host. Intra-footpad growth of 10⁴ tumor cells was equally inhibited by 4 × 10⁴ admixed LM in normal or LM-immune mice indicating that concomitant or prior immunity to LM was equally effective in suppressing tumor growth. Development of cellular immunity to viable LM was required for tumor rejection. Mice prevented from developing anti-LM immunity by inoculation of dead bacteria were also incapable of inhibiting tumor growth. Further, a functionally active reticuloendothelial system was essential for nonspecific inhibition of tumor development as temporary "paralysis" of the reticuloendothelial system by a prior injection of 10⁴ heat-killed LM reduced the effectiveness of LM-mediated tumor suppression. Histological examination of LM or LM tumor-injected sites revealed a stepwise development of LM-mediated inflammatory reaction of delayed type associated with gradual degeneration of the adjacent tumor cells.

INTRODUCTION

The tumor-inhibitory effect of LM has been described for a number of experimental tumors in mice (2, 28, 30) and guinea pigs (3). An apparent requirement for the immunotherapeutic potential of LM, Bacillus Calmette-Guérin (4, 6), Brucella abortus (27), or Salmonella (24) is the acquisition of cellular or infectious immunity (14) by the host to the bacterial agents. This idea was substantiated by recent studies in vitro (28, 31) which showed that cellular interaction between specifically sensitized T-cells and adherent cells resulted in release of mediators (29) inhibitory to tumor cell proliferation. Elimination of either cell population abrogated the inhibitory effect. The present report demonstrates that procedures which lead to "paralysis" of the RES or prevent induction of antigen-reactive T-cells result in elimination of tumor suppression by LM in vivo.

MATERIALS AND METHODS

Animals. Male and female inbred A/He mice, 6 to 10 weeks old, were used in all experiments.

Bacterial Cultures and Immunization. LM strain EGD was maintained in virulent state by continuous passage in mice. Spleens from injected mice 3 to 4 days after infection were homogenized and inoculated into tubes of trypticase soy broth. Cultures were grown for 18 hr at 37°, and the bacteria were recovered by centrifugation at 1000 × g. The bacterial pellet was washed in 0.9% NaCl solution, resuspended, and counted in a Petroff-Hausser bacterial counter and phase-contrast microscope. An inoculum was prepared containing the desired number of viable bacteria per ml. The number of the viable bacteria was confirmed by plating on brain-heart infusion agar. Bacteria were killed by placing the prepared inoculum in boiling water for 15 to 20 min. Mice were immunized with 2 to 5 × 10⁴ viable or heat-killed LM in 0.1 ml of 0.9% NaCl solution.

Bacterial Enumeration in Spleen and Liver of Mice. Organs were removed from LM-injected animals aseptically and homogenized in 0.9% NaCl solution using a motorized ground-glass pestle. Samples were kept at 2-4° until all animals had been processed. The homogenates were further dispersed by mechanical vibration on a Vortex mixer into tubes containing 5 to 10 glass beads. The volume of the uniform suspension was measured and 0.1 plated in duplicate on dry brain-heart infusion agar plates after serial dilution in 0.9% NaCl solution. Colonies were counted 24 to 48 hr later.

Transplantable Spindle Cell Sarcoma. A/He mice received i.p. injections of 2 mg 3-methylcholanthrene in 0.1 ml triacetanoin. One of the tumor nodules (S-40) that developed 3 months later has been serially transplanted since and used in the experiments.

Preparation of Single-Tumor-Cell Suspension. Approximately 1 g tumor tissue excised from the surrounding tissue was minced into small fragments, washed 2 to 3 times, and placed in 50 ml of Medium 199 (Grand Island Biological Co., Grand Island, N. Y.) containing 0.25% Pronase (Calbiochem, Los Angeles, Calif.) and 40 μg RNase (Sigma Chemical Co., St. Louis, Mo.) per ml. Enzymatic digestion of tumor was allowed to proceed for 60 min on a magnetic stirrer at room temperature. The dispersed tumor cells were filtered through 3 layers of gauze, washed 3 times in Medium 199, counted in a hemocytometer, and resuspended in Medium 199. Cell viability was 95% or more as determined by trypan blue dye exclusion.

Inoculation and Measurement of Tumor Growth. The desired number of tumor cells suspended in 0.01 ml of medium was injected into 1 footpad, and an equal volume of Medium 199 was injected into the contralateral or control footpad. In some experiments equal volumes of tumor cells.
and viable or heat-killed LM inoculum were mixed and injected together in a total volume of 0.02 ml. Tumor growth was measured along 1 diameter using vernier calipers (Gio-
gau's Vernier Caliper No. 12) and calculated as total volume using the formula: tumor volume = \( 4/3 \pi r^3 \) (\( r \) = radius). Tumor size was expressed as the difference between the experimental and control footpad. Results were expressed as mean tumor volume for all members of each experimental group.

**Histological Preparations.** Footpads or tumors were cut out and fixed in 10% NaCl-buffered formalin. Thin sections were stained with hematoxylin and eosin or Brown-Brenn gram stains.

**RESULTS**

**Tumor Cell Histology.** The tumor was composed of masses of neoplastic spindle-shaped cells containing large hyperchromatic axial or elongated nuclei with 1 or more nucleoli. There were many bundled and twisted cells with whorl formation. Large numbers of mitotic figures and multinucleated giant cells were observed. The cells had scanty cytoplasm and appeared fibrillar, resembling fibroblastic cells. Areas of necrosis and hemorrhage were also observed.

**Tumor Dose Titration.** Groups of normal A/He mice received injections in the footpad of \( 10^3 \), \( 3 \times 10^3 \), \( 10^4 \), \( 10^5 \), or \( 4 \times 10^4 \) S-40 tumor cells. The inoculated footpads were inspected, and tumor growth was measured at regular intervals. The results of this experiment are presented in Chart 1 and indicate that tumor behaved as a function of tumor cell dose injected. The number of successful tumor grafts and tumor size increased while the latent period for appearance of the tumors decreased with increasing tumor cell dose.

**Suppression of Tumor Growth at Site of Inoculation with LM-Tumor Cell Mixture.** A group of A/He mice were immunized with \( 3 \times 10^8 \) LM i.v. through a tail vein. Two weeks later these and a group of normal mice were challenged in 1 footpad with a mixture of \( 4 \times 10^4 \) LM and \( 10^4 \) tumor cells in a total volume of 0.02 ml. A group of normal mice received tumor cells alone. Emerging tumors were measured regularly. These results are presented in Table 1 and indicate suppression of tumor growth at the site of LM challenge. Prior immunity to LM did not necessarily enhance host resistance to tumor development. Tumor growth in normal or LM-immune animals challenged with LM and tumor cells was equally and significantly suppressed as compared to control mice given tumor cells alone. Statistical analysis using Student's t test indicated no significant differences in tumor sizes on Day 38 when comparing Groups 1 and 2 (\( p < 0.50 \)); however, \( p \) values comparing Group 1 versus Group 3 and Group 2 versus Group 3 were \( p < 0.005 \) and \( p < 0.01 \), respectively, i.e., significantly different.

**Lack of Tumor Suppression in the Absence of Acquired Immunity to LM.** The relationship between acquired cellular immunity to viable LM and nonspecific suppression of tumor cells was investigated. The results (Table 2) indicated that specific host immunological reactivity to LM was a required component of the antitumor effect. To demonstrate this requirement, 2 groups of normal mice were immunized in the footpad with \( 5 \times 10^3 \) viable or heat-killed LM, respectively. Ten days later, 5 mice from each group were challenged with \( 5 \times 10^3 \) viable LM in the footpad, and 72 hr later the total number of bacteria in the spleen of each animal was determined. An average of 1.3 \( \times 10^8 \) organisms were isolated from each spleen of mice immunized with viable LM, while an average of \( 5 \times 10^7 \) bacteria were isolated from each spleen of mice initially treated with heat-killed LM. Therefore, cellular immunity (14) was only acquired by mice previously exposed to a viable dose of LM. This procedure was then used to determine the requirement of host cellular immunity to LM on tumor inhibition. Six normal mice were challenged in the footpad with \( 5 \times 10^3 \) heat-killed LM in admixture with \( 10^4 \) S-40 tumor cells. Tumor growth in this group of mice, a group of controls inoculated with S-40 cells alone, and a 3rd group inoculated with viable LM and tumor cells was monitored. The results (Table 2) show that tumor growth was similar in Groups 1 and 2 where tumor cells were injected with heat-killed LM or no LM at all. On the other hand, viable LM markedly inhibited development of tumors as expected (Group 3).

**The Effect of “Reticuloendothelial Blockade” on Tumor Suppression.** Inhibition of systemic immunity to LM and its effect on tumor suppression were further tested after partial “paralysis” or “blockade” of the host's RES. This blockade was achieved by an i.v. injection of \( 10^8 \) heat-killed LM in a group of experimental animals used in the following study.
Twenty-four hr after injection of the blocking dose, this group of mice and a group of normal controls received a challenge dose of $5 \times 10^5$ viable LM in 1 footpad. On Days 3, 5, and 7 following this treatment, LM was isolated from the liver and spleen of groups of 5 mice from the experimental and control mice. The results (Chart 2) clearly demonstrate the suppressed capacity of the blocked group of mice to clear the challenge dose of viable LM from their spleen and liver. During the 1st few days clearance of viable LM was apparently preempted by the massive numbers of circulating and sequestered dead bacteria. By Day 5, these animals had essentially assumed a clearance pattern characteristic of their normal cohorts. By Day 7, these mice had also mounted an immunological response and were clearing the challenge dose of the viable organisms; however, the number of isolated bacteria from livers and spleens of these mice was still 10 times as great as the number isolated from similar organs of the control mice.

The effect of depressed reticuloendothelial activity on nonspecific suppression of tumor growth was investigated by determining the effect of an initial i.v. injection of $10^8$ heat-killed LM and subsequent challenge with $10^4$ tumor cells admixed with $3 \times 10^4$ viable LM 24 hr later. The results of 1 of 2 similar experiments are presented in Table 3. A significant suppression of antitumor effect occurred as a result of pretreatment with the blocking dose of LM (compare Groups 1 and 2). This was reflected in both tumor size and number of successful tumor grafts at each time point. The participation and importance of the RES in resisting tumor growth become apparent by comparing Groups 3 and 4. Although less significant than LM-mediated tumor suppression, the definite advantage of intact RES on arresting tumor proliferation is well demonstrated. Comparison of Groups 1 and 3 indicates LM-mediated tumor suppression despite the compromised RES of the blocked animals.

**Histological Observations.** Histological examination of LM tumor cell-injected footpads showed intense deep and superficial inflammatory reactions at 72 hr. Mononuclear and histiocytic cells had infiltrated the reaction site and the beginnings of a granulomatous reaction could be observed. The tumor cells were necrotic, and residual numbers of LM were seen. At 6 days, a more chronic form of the above reaction had developed; large numbers of mononuclear cells had entered the area. A delayed-type hypersensitivity reaction and macrogranuloma had developed and only a few tumor cells were seen. A similar histological picture developed as a result of LM injection alone. On Day 6, injection of tumor cells had evoked a moderate allergic response of delayed type in the presence of massive numbers of tumor cells.
Effect of RES blockade in nonspecific suppression of tumor growth

Treatment of mice

<table>
<thead>
<tr>
<th>Pretreatment with heat-killed LM</th>
<th>Challenged with</th>
<th>Mean tumor size on Day (cu mm ± S.E.)a</th>
</tr>
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<tbody>
<tr>
<td>Viable LM (3 × 10^4)</td>
<td>Tumor Cells (10^4)</td>
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<td>Group No.</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>1</td>
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a Mean tumor size ± S.E. or groups of 5 to 8 mice given injections of viable LM-tumor mixture (Groups 1 and 2) or tumor cells alone pretreated with (Groups 1 and 3) or without (Groups 2 and 4) 10^8 heat-killed LM.

Numbers in parentheses, number of mice with tumors per number of mice inoculated.

DISCUSSION

Intra-footpad injection of normal mice with LM significantly retarded the growth of a murine fibrosarcoma at the same site (Table 1). Tumor growth at the site of either a primary or secondary infection with LM was equally and significantly depressed compared to controls given tumor cells alone. The inhibitory effect of LM was reflected in the latent periods for the appearance of the tumors and the number of successful tumor takes.

Two lines of evidence demonstrated that immunity to LM was a prerequisite for tumor rejection. First, injection of mice with heat-killed LM did not provide immunity either against a viable challenge dose of LM or protection against an admixed dose of tumor cells (Table 2). These results are comparable to others using Bacillus Calmette-Guérin. Guinea pigs (6) and mice (4) prevented from developing cellular immunity to myobacterial antigens by treatment with cortisone acetate or antithymocyte serum could not effect the regression or suppression of their respective tumors. Both immunosuppressive agents apparently influence a subclass of lymphocytes (16, 20) which mediate cellular immunity and resistance to intracellular infection by LM. Second, animals rendered temporarily unresponsive to LM antigens by prior injection of a large dose of heat-killed LM were significantly suppressed in their anti-LM immunity (Chart 2) and tumor-suppressive activity (Table 3, compare Groups 1 and 2). The rate of tumor growth and number of tumor takes were significantly enhanced in the heat-killed LM-pretreated animals. A prior i.v. injection of Corynebacterium parvum similarly reduced the marked inhibition of tumor growth observed after intratumor injection of C. parvum (25). Several possible conclusions may be derived from these experiments. The large dose of dead LM may have depressed development of delayed-type hypersensitivity and cell-mediated immunity which are characteristics of host response to viable LM (14) either by antibody-mediated feedback inhibition (13, 17) or possibly by induction of regulatory suppressor cells (1). Alternatively, antibody to killed LM may have prevented engulfment of tumor cells by activated macrophages. Keller (10) observed that the IgG2 component of rat antiserum to Nippostrongylus brasiliensis prevented the in vitro phagocytosis of an antigenically unrelated tumor cell by activated macrophages. At the inductive or sensitization phase to LM, macrophages are required to

Table 3

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Numbers in parentheses, number of mice with tumors per number of mice inoculated.

Number of mice with tumors per number of survivors. Data represent tumor size for remaining animals.
process and present the bacterial antigen to antigen-reactive cells (26). Macrophages are also required at the effector phase of tumor cell destruction to release toxic materials (8), become cytotoxic upon activation (9, 23), or cooperate with T-cells (5, 29, 31) to eradicate tumor cells. The preoccupation of the paralyzed animals to clear the dead organisms in some fashion may incapacitate them to carry on with any of these postulated functions and thus result in tumor growth.

The mechanism(s) of LM-mediated nonspecific inhibition of tumor growth in vivo are not clear. Certainly, it seems that macrophage- and antigen-reactive T-cells are required for induction of cell-mediated immunity to the bacterial antigens and that such a host response is obligatory for the observed antitumor effect. In vitro (29, 31), it was determined that macrophage-T-cell interaction by both peritoneal and spleen cells occurred prior to release of soluble factor(s) cytolytic for the B-16 melanoma. Cellular mediators of anti-Listerial immunity comprise a rapidly induced (20, 21, 32) population of short-lived thymus-derived lymphocytes which enter foci of infection where they influence macrophage accumulation and activation (12, 15, 19, 21, 22). It is, therefore, quite conceivable that such immune hosts can react to a secondary challenge with LM (30) or during the early stages of infection to residual bacterial cells at the injected site, i.e., the footpad. This sequence of events may then promote destruction of bystander tumor cells through release of toxic soluble factors. Histological examination of LM tumor sites did reveal presence of infiltrating mononuclear cells adjacent to tumor cells and LM, suggesting the possibility of the outlined events during tumor cell destruction. On Day 6, injection of tumor cells alone had evoked a moderate allergic response of delayed type in the presence of massive numbers of tumor cells. The contribution of this response to tumor destruction can at best be very limited since, by this time, only a few tumor cells had escaped the LM-mediated inflammatory response, the majority having been destroyed. Since LM-mediated tumor suppression is a transitory phenomenon with eventual recurrence of most tumors, the tumor-induced reaction must then be relatively ineffective in the eradication of the residual tumor cells.

A direct cytotoxic function by the accumulated macrophages in vivo remains speculative at present; however, degenerating tumor cells in close association with macrophages and histiocytes were observed upon intratumor injection of Bacillus Calmette-Guerin (7). This phenomenon, however, may represent an immunologically specific reactivity against the tumor-specific antigens. In vitro (29, 31) purified macrophages from 12- to 13-day LM-immune mice did not exert any direct tumor-inhibitory effect on B-16 melanoma, unless combined with specifically sensitized T-cells. The in vivo data suggest that similar interaction may occur during tumor cell rejection, although alternate mechanisms of action cannot be ruled out.

Nonspecific stimulation of host immunological capacity can be quite effective in immunotherapy of certain types of neoplasia, particularly those of the skin. Cancer patients with malignant melanoma (18), adenocarcinoma of the breast, mycosis fungoides, and reticulum cell sarcoma (11) have received the most benefit offered by this type of therapy. Availability of experimental models can help in a clearer understanding of the mechanisms involved and extension of immunotherapy to sites other than the skin.

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REFERENCES

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