The Effect of a Single and Repeated Administration of Corynebacterium parvum on Bone Marrow Macrophage Colony Production in Syngeneic Tumor-bearing Mice

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SUMMARY

Administration of Corynebacterium parvum to tumor-bearing animals results in a greater augmentation of bone marrow macrophage colony production than do similar inoculations to non-tumor-bearing mice. Comparison of a single injection of C. parvum with one of Bacillus Calmette-Guérin in tumor-bearing mice indicates that both agents can stimulate macrophage colony production, to a similar extent. A second inoculation of C. parvum was able to effect a significant augmentation in bone marrow macrophage production, whereas a similarly administered second inoculation of B. Calmette-Guérin failed to elicit such a response. The possible application of these results to elucidate an optimum therapeutic schedule for the use of immunopotentiators is discussed.

INTRODUCTION

Various strains of anaerobic coryneforms are notable for their tumor growth-inhibiting properties (9, 10, 13, 19, 24) and their capacity to function as potent immunopotentiators (6, 20, 21). Although no single explanation is available to account for its mechanism of action, considerable evidence has accumulated that implicates activation of macrophages as an important factor in C. parvum-elicited immunopotentiation. Such macrophages are of importance in the regulation of C. parvum-induced stimulation of B-cell function (15, 16) and inhibition of T-cell function (14, 22), as well as in the augmentation of host resistance to infection (1, 7). In addition, it has been postulated that activated macrophages are responsible for the induction of lymphocyte trapping, a mechanism that can be markedly amplified and prolonged by the administration of C. parvum (11).

Attempts to elucidate a specific cell type involved in C. parvum-mediated inhibition of tumor growth have implicated activated macrophages. Evidence for their importance has been provided indirectly by the demonstration that C. parvum inhibition of tumor growth can probably proceed without dependence on T- or B-cell function, since C. parvum tumor-growth inhibition has been shown to occur in "B" mice whose T-cells have been severely depleted (25) or in mice treated with antilymphocyte serum (5). In addition, it has been demonstrated that C. parvum-induced tumor-growth inhibition can be improved by the administration of B-cell-depressant cytostatic agents (9) and appears to be unaffected by different levels of humoral antibody production (3). More direct evidence is furnished by Halpern et al. (13) who have shown that peritoneal macrophages from animals treated with C. parvum can inhibit the in vitro proliferation of syngeneic tumor cells.

In view of the importance of macrophage mediation in C. parvum adjuvant activity, a study was undertaken in this laboratory to evaluate the effects of single and repeated administrations of C. parvum on bone marrow macrophage colony production in mice with established growing tumors. The findings are compared with those obtained when BCG was similarly used in tumor-bearing animals and with those previously obtained and reported by us following administration of C. parvum to normal mice.

MATERIALS AND METHODS

Plan of Investigation. Two experiments were performed. In Experiment 1, the effect of a single injection of C. parvum on bone marrow macrophage colony production was determined in animals bearing growing syngeneic mammary adenocarcinomas. Four groups of animals were utilized. Tumors were implanted on Day zero in mice in Groups 1 and 2, whereas mice in Groups 3 and 4 served as non-tumor-bearing controls. Animals in Groups 1 and 3 received 0.2 ml or 1.4 mg, dry weight, of C. parvum, and those in Groups 2 and 4 received 0.2 ml of 0.9% NaCl solution. All injections were administered s.c. into the left hind leg between the tumor and regional (popliteal and inguinal) lymph nodes at a time corresponding to Day 14 of tumor growth. Macrophage assays were performed 7 days after injection (at Day 21 of tumor growth). There were 3 mice in each group, and the experiment was carried out a total of 3 times.

Experiment 2 was designed to ascertain the effect of a 2nd inoculation of C. parvum or BCG on bone marrow macrophage colony production in animals bearing syngeneic mammary adenocarcinomas. Six groups of animals were used. Tumors were implanted on Day zero in mice in Groups 1 to 5, whereas...
those in Group 6 were non-tumor-bearing mice. Animals in Group 1 received 1.4 mg \( C. \) parvum on Days 10 and 24, those in Group 2 received 1 mg BCG on Days 10 and 24, and those in Group 3 received 0.1 ml 0.9% NaCl solution on Days 10 and 24. Mice in Group 4 were inoculated with 0.1 ml 0.9% NaCl solution on Day 10 and a single injection of \( C. \) parvum on Day 24; those in Group 5 received 0.1 ml 0.9% NaCl solution on Day 10 and a single injection of BCG on Day 24, while those in Group 6 received 0.1 ml 0.9% NaCl solution on Days 10 and 24. All injections were administered s.c. in the left hind leg between the tumor and regional lymph nodes (popliteal and inguinal). Macrophage assay was carried out on Day 31 of tumor growth (7 days after the last injection). Tumor diameters were measured on Days 10 and 31 of tumor growth. There were 3 animals in each group, and the experiment for macrophage assay was carried out a total of 5 times, whereas tumor-growth diameters were obtained from 6 experiments. Results are expressed as macrophage colony counts/plate (mean ± S.D).

Mice. Adult male C3HeB/FeJ mice (The Jackson Laboratory, Bar Harbor, Maine) were utilized when 8 to 12 weeks old.

Tumor. The tumor used was a spontaneous syngeneic mammary adenocarcinoma that originated in a C3H female and was maintained by transfer in female mice. Tumor implantation consisted of the trocar s.c. transfer of a 1- to 2-mm fragment of tumor into the left hind leg distal to the popliteal node. The greatest tumor diameter was measured, and the same diameter was utilized for all subsequent measurements. Prior to the commencement of therapy, tumors were measured and animals were distributed in a manner that ensured that each treatment group contained tumors of equivalent diameters.

\( C. \) parvum. \( C. \) parvum, Culture 6134 (Batch PX365A), was obtained from Burroughs Wellcome Company, Research Triangle Park, N. C. Preparations consisted of 7 mg, dry weight, per ml formalin-killed organisms suspended in 0.9% NaCl solution (containing 0.01% Thiomersol).

BCG. BCG was obtained from the Research Foundation, Chicago, Ill. Preparations that consisted of \( 3.62 \times 10^8 \) lyophilized live organisms (15 mg, dry weight) were reconstituted in 1.5 ml sterile water and used immediately.

Bone Marrow Macrophage Assay. A modification of the method of Bradley and Metcalf (4), described in detail elsewhere, was used (2). All animals were killed by cervical dislocation and were immediately immersed in antiseptic solution, and 1 femur was promptly removed by sterile technique from each of 3 mice in a group. Marrow cells were harvested. An aliquot of the pooled marrow cell suspension was counted and diluted so that there were \( 1 \times 10^6 \) cells/ml. One and one-half ml of the suspension were added to 9.6 ml of methylcellulose solution containing 2.25 ml of horse serum. One ml of the resulting suspension (containing \( 1.1 \times 10^5 \) cells) was placed in each of 10 tissue culture dishes (35 x 10 mm, Falcon Plastics, Oxnard, Calif.). Plates were incubated at 37\(^\circ\) in a 10% CO\(_2\) atmosphere with 100% humidity for 7 days. After that period of incubation, discrete colonies of cells were found, as well as small clusters of fewer cells. Evidence has been provided previously (2) that indicates that such cells are macrophages. Identification marks on plates were replaced by a code to ensure objectivity of the colony counts. Only groups of 25 cells or more arranged in a colony configuration were counted. In general, the greater the colony count, the larger the colonies.

Statistical Analysis. Student's \( t \) test was used, with a level of significance of 0.01.

RESULTS

Results of Experiment 1 (Table 1) demonstrated that the presence of an established growing tumor had no stimulatory effect on bone marrow macrophage colony production at 21 days of tumor growth (Group 2), with levels being observed that were no greater than non-tumor-bearing controls (Group 4). However, if \( C. \) parvum was administered to an animal with a tumor at 14 days of growth (Group 1), significantly greater bone marrow macrophage colony production was achieved at 21 days than when a similar inoculation was administered to a non-tumor-bearing animal (Group 3).

Results of Experiment 2 (Table 2) indicated that a single administration of either \( C. \) parvum or BCG at 24 days of tumor growth increased bone marrow macrophage colony production (Groups 4 and 5) to a similar degree. As in Experiment 1, the presence of a growing tumor no longer had any bone marrow macrophage-stimulating properties at 31 days (Group 3), and levels obtained were no different from those of non-tumor-bearing controls (Group 6). Two inoculations of \( C. \) parvum administered on Days 10 and 24 of tumor growth (Group 1) achieved a much greater bone marrow macrophage colony production than did 2 similarly administered doses of BCG (Group 2). Two inoculations of \( C. \) parvum (Group 1) had a considerably greater bone marrow macrophage stimulation than did a single inoculation given at

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor(^a)</th>
<th>Treatment</th>
<th>Day given</th>
<th>Macrophage colony count/plate</th>
<th>( p ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>( C. ) parvum</td>
<td>14</td>
<td>202.1 ± 19.4(^b)</td>
<td>Group 1–2, 3, 4 = &lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>0.9% NaCl solution</td>
<td>14</td>
<td>127.5 ± 24.7</td>
<td>Group 2–3 = &lt; 0.01</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>( C. ) parvum</td>
<td>14</td>
<td>150.0 ± 24.2</td>
<td>Group 3–4 = &lt; 0.01</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>0.9% NaCl solution</td>
<td>14</td>
<td>128.6 ± 20.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Implanted on Day 0.  
\(^b\) Mean ± S.D. of 3 experiments, 20 observations.
DISCUSSION

Previous investigations conducted in this laboratory have demonstrated that the presence of an established growing syngeneic tumor is capable of augmenting the production of bone marrow macrophage colonies (2). However, this stimulus was transient and, at Day 14 of tumor growth, was no longer effective in spite of the continuing presence of progressively increasing levels of tumor antigen. The present results confirm those findings in that no stimulatory effect was noted at Day 21 of tumor growth. When C. parvum was administered after 14 days of tumor growth, i.e., at a time when stimulation of macrophage precursors would have been expected to have ceased, a synergistic augmentation in macrophage colony production was noted. Although available tumor antigen was not in itself still acting as a stimulus to macrophage production, it probably had a sensitizing effect on macrophage precursors permitting levels of stimulation by C. parvum greater than that occurring when C. parvum was administered to normal animals. If, as previously speculated by us (2), the transient duration of bone marrow macrophage colony production by tumor antigen is due to a negative feedback inhibition, it might be postulated that C. parvum functions by interrupting such an inhibitory mechanism as well as by independently stimulating available bone marrow macrophage precursors, thus effecting a synergistic augmentation. These results are similar qualitatively to those acquired when BCG was utilized in place of C. parvum (8).

Recently, findings obtained in this laboratory from experiments utilizing single and repeated doses of C. parvum or BCG in non-tumor-bearing animals have been reported (23). Those results indicated that a 2nd inoculation of C. parvum, administered at a time when stimulation from the 1st inoculation was terminating, was able to effect a synergistic augmentation in macrophage colony production, exceeding levels obtained from a single administration. In contrast, that phenomenon could not be achieved by means of a 2nd inoculation of BCG, which appeared to be incapable of restimulating bone marrow cells. These findings obtained in tumor-bearing animals indicate that the synergistic augmentation of bone marrow stimulation achieved by a repeated dose of C. parvum is fully functional in tumor-bearing animals, and the presence of tumor antigen does not appear to induce significant alteration. As in normal animals, 2 administrations of BCG were ineffective in significantly restimulating bone marrow cells. It is of interest that the group of animals receiving 2 administrations of C. parvum was the one that demonstrated the most marked macrophage colony production and the only one that showed a small but significant suppression of tumor growth.

Determination of patterns of bone marrow macrophage stimulation may represent a useful method to elucidate an optimum therapeutic regimen for the administration of various immunological adjuvants. If a correlation between bone marrow macrophage stimulation and inhibition of tumor growth can be substantiated, then utilization of the presently employed technique for the screening and monitoring of prospective immunotherapeutic agents would have merit. Moreover, it would provide a method for determining the dose and schedule of administration that would be capable of maintaining a sustained elevated level of bone marrow macrophage stimulation. This would be of particular pertinence when immunostimulants are utilized together with myelosuppressive chemotherapeutic agents (17, 18). Under such circumstances, an optimum bone marrow-protective effect could be achieved as a result of maximum marrow stimulation. From the present findings, it appears that repeated administrations of C. parvum are apt to be more effective than is administration of BCG.

ACKNOWLEDGMENTS

The expert technical assistance of Morton Levine in culturing macrophages, Pat Szuch in preparing animals, and Elizabeth Saffer in aiding the research, is gratefully acknowledged. We are indebted to Dr. John K. Whisnant, Burroughs Wellcome Company, for furnishing us with C. parvum for these studies.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor</th>
<th>Treatment</th>
<th>Days given</th>
<th>Macrophage colony count/plate</th>
<th>p values on colony counts &lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>C. parvum</td>
<td>10 and 24</td>
<td>265.9 ± 26.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Groups 1–2, 3, 4, 5, 6</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>BCG</td>
<td>10 and 24</td>
<td>168.9 ± 21.6</td>
<td>Group 2–3, 6</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>0.9% NaCl solution</td>
<td>10 and 24</td>
<td>118.7 ± 32.7</td>
<td>Group 3–4, 5</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>C. parvum</td>
<td>24</td>
<td>181.6 ± 21.1</td>
<td>Group 4–6</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>BCG</td>
<td>24</td>
<td>161.7 ± 32.3</td>
<td>Group 5–6</td>
</tr>
<tr>
<td>6</td>
<td>−</td>
<td>0.9% NaCl solution</td>
<td>10 and 24</td>
<td>124.5 ± 24.4</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Implanted on Day 0.
<sup>b</sup> Mean ± S.D. of 5 experiments, 26 observations.
<sup>c</sup> p value, Group 1 versus 3 = < 0.01, 31 days, 18 animals per group, mean ± S.D. of 6 experiments.
REFERENCES

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