Immunological Enhancement of Leukemia L1210 by Corynebacterium parvum in Allogeneic Mice

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SUMMARY

The effect of Corynebacterium parvum on the immune response of C57BL/6 mice (H-2b) to the allogeneic leukemia L1210 (H-2a) was investigated. Mice were either left untreated or given C. parvum i.v. or i.p. in various dosages. Seven days later they were challenged with 2.5 to 10 x 10^6 live L1210 cells i.p. Control animals almost always rejected the challenge. In contrast, most mice pretreated with either 1.0, 0.5, or 0.25 mg of C. parvum i.v. and 1.0 or 0.5 mg i.p. exhibited enhanced growth of leukemia L1210 as indicated by gross ascites and significantly greater weight gain. This sometimes progressed to the death of the animal, but more often regressed after several days. Spleen cell-mediated cytotoxicity to alloantigens, evaluated in vitro by release of ^51Cr from P815Y (H-2d) target cells, was significantly decreased in the mice pretreated with either 1.0 or 0.5 mg of C. parvum i.v. or 0.5 mg of C. parvum i.p. This suppression could not be reversed by reduction of the concentration of macrophages in the spleen cell suspensions. Complement-dependent cytotoxic antibody, measured by release of ^51Cr from L1210 cells, was profoundly suppressed in mice pretreated with C. parvum i.v. in dosages ranging from 1.0 to 0.1 mg. These data suggest an immunological basis for the enhanced growth of leukemia L1210 caused by C. parvum at these schedules.

INTRODUCTION

Killed suspensions of Corynebacterium parvum have been demonstrated to be effective in the prevention and treatment of a variety of animal tumors (12, 15, 20, 22, 24). Preliminary results suggest that C. parvum may have some activity in human tumors as well (10). Its mechanism of action is thought to be related to generation of activated, tumoricidal macrophages but has not been well delineated (7, 16).

We decided to investigate the immunological effects of C. parvum in an allogeneic murine tumor system. It was expected that C. parvum would have easily discernible adjuvant effects that could be detected by in vitro tests of cell-mediated and humoral immunity. Instead, we found unexpectedly that at our dosage schedules C. parvum caused enhancement of tumor growth and a concomitant depression of several immunological parameters.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2b) female mice (The Jackson Laboratory, Bar Harbor, Maine) were used when 6 to 10 weeks old. Tumors. Leukemia L1210 (H-2a) was propagated by weekly passage in C57BL/6 x DBA/2 (hereafter called B6D2F1) mice. P815Y mastocytoma (H-2d) was carried in suspension cultures in Fischer’s medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% horse serum; it was periodically passed in B6D2F1 mice to ensure retention of transplantation antigens. C. parvum. Lot PX289, that contained 0.01% thiomersal as a preservative, was obtained from the Burroughs Wellcome Co., Research Triangle Park, N. C. It was diluted with 0.9% NaCl solution so that the volume injected was 0.10 to 0.20 ml. Corynebacterium granulosum was obtained from the Pasteur Institute, Paris, through the courtesy of Dr. N. Dimitrov.

Experimental Design. Mice were either left untreated or given C. parvum i.v. or i.p. in various dosages. Seven days later, all mice were given i.p. injections of 2.5 x 10^6 live L1210 cells. Ten days after tumor challenge, all mice were bled to obtain serum and were sacrificed for the spleen cell assay.

Spleen Cell-mediated Cytotoxicity. A modification of the method of Brunner et al. (4) was used. Spleen cell suspensions were prepared by disrupting spleens in glass tissue grinders. The suspensions were allowed to stand for 2 min so that debris could settle and be removed. They were then washed twice in cold Fischer’s medium and resuspended in Fischer’s medium with 10% heat-inactivated horse serum. The suspensions were adjusted to the desired concentration of live nucleated cells by counting in a Coulter counter (Coulter Electronics, Becton, Md.) and by staining with trypan blue.

P815Y cells were labeled by adding 100 to 200 µCi of ^51Cr (0.1 to 0.2 ml) (New England Nuclear Corp., Boston, Mass.)
to $5 \times 10^6$ cells in 1.0 ml of Fischer’s medium with 10% horse serum. The mixture was incubated for 60 min at 37°, washed 4 times, and then adjusted to a concentration of $50 \times 10^6$/ml.

Various numbers of spleen cells in 1.0 ml of Fischer’s medium with 10% horse serum were mixed with $5 \times 10^4$ (0.1 ml) $^{51}$Cr-labeled P815Y cells in 17- x 100-mm plastic tubes (Falcon Plastics, Oxnard, Calif.). Ratios of effector to target cells varied from 100:1 to 10:1. The tubes were centrifuged at $250 \times g$ for 2 min and then incubated at $37^\circ$ in an atmosphere of 5% CO$_2$ in air for 6 to 18 hr. After the incubation period, 1 ml of cold Fischer’s medium was added, as was 0.1 ml of goat RBC (Difco Laboratories, Detroit, Mich.) in 1% rabbit serum, the latter to stabilize the pellet. The tubes were centrifuged at $300 \times g$ for 15 min and then the supernatant fluids were removed. Each pellet and supernatant fluid was counted in a gamma counter. For each sample, the percentage of $^{51}$Cr release was calculated by dividing the number of counts in the supernatant fluid by the sum of the number of counts in the supernatant fluid and the pellet. Specific release was calculated by subtracting the spontaneous loss of $^{51}$Cr by mastocytoma cells alone (usually 15 to 30%).

**Removal of Adherent Cells.** Nucleated spleen cells (75 to 100 $\times 10^6$) in 10.0 ml of Fischer’s medium with 10% heat-inactivated horse serum were incubated on 150- x 25-mm plastic dishes (Falcon Plastics) for 45 min at $37^\circ$ in an atmosphere of 5% CO$_2$ in air. The plates were rotated 50 times and nonadherent cells were removed. These nonadherent cells were then treated a 2nd and 3rd time in the same manner. Following the 3rd incubation, the nonadherent cells were centrifuged, resuspended, and counted as outlined above.

**Cytotoxic Antibody.** A modification of the method of Herberman and Oren (8) was used. L1210 cells were used as targets, because in this assay they released more $^{51}$Cr than P815Y. L1210 cells (1 $\times 10^6$) labeled with $^{51}$Cr (5,000 to 10,000 cpml in 0.1 ml Fischer’s medium with 10% heat-inactivated horse serum were added to 12- x 75-mm plastic tubes (Falcon Plastics) that contained 0.1 ml of serial dilutions of test sera. The tubes were incubated at $37^\circ$ for 15 min. Then 0.1 ml of a 1:3 dilution of guinea pig complement was added to each tube, and the tubes were incubated at $37^\circ$ for 30 additional min. Following this incubation, 2.0 ml of ice-cold medium was added to each tube, and the tubes were centrifuged at $300 \times g$ for 15 min. A 1.0-ml aliquot of supernatant fluid was removed and counted in a gamma counter. The tube containing the pellet and the remaining supernatant fluid was also counted. The number of counts in each aliquot of supernatant fluid was divided by the sum of the counts in this aliquot and the counts in the pellet and remaining supernatant fluid. The result was multiplied by the dilution factor of 2.3 to give percentage of $^{51}$Cr release. Spontaneous release (L1210 cells with medium and complement) was approximately 5%.

**RESULTS**

**In Vivo Observations.** Nearly all of the control mice were able to reject a challenge of $2.5 \times 10^6$ L1210 cells (Table 1).

Minimal, transient ascites was sometimes noted, but 10 days after challenge these mice appeared to be normal. Large, activated macrophages predominated in the peritoneal fluid. In contrast, mice pretreated with *C. parvum* 7 days before tumor challenge exhibited progressive growth of leukemia L1210 as indicated by gross ascites and weight gain, the ascitic fluid consisting mainly of L1210 cells.

Enhancement was observed in almost all mice pretreated with 1.0 or 0.5 mg *C. parvum* i.v. or i.p. and in 5 of 9 pretreated with 0.25 mg i.v., but was not seen in mice pretreated with 0.10 *C. parvum* i.v. or 0.25 mg i.p. *C. granulosum* (0.5 mg i.v.) was as effective in producing enhancement as *C. parvum*. No morbidity or mortality was observed in mice given 1.0 mg *C. parvum* i.v. 1 day after tumor inoculation, challenged with only $5 \times 10^6$ L1210 cells 7 days after *C. parvum*, or those given 0.9% NaCl solution with thiomerosal i.v.

*C. parvum* pretreatment did not result in increased mortality from $2.5 \times 10^6$ L1210 cells during the 10 days of observation, except in the mice given 0.5 mg i.v. We considered the possibility that the larger number of deaths in this group could have been due to a less resistant batch of mice in early experiments, since this was not found in subsequent experiments. However, in order to more thoroughly evaluate the natural history of the L1210 enhancement, $10 \times 10^6$ L1210 cells were given i.p. to 22 *C. parvum*-pretreated mice (0.5 mg i.v. 7 days before tumor challenge) and to 20 normal mice. The mice were weighed at regular intervals, weight gain reflecting accumulation of malignant ascites. They were observed until death or until the weights returned to near prechallenge levels. The controls all rejected the tumor challenge. These animals experienced a mean weight gain of $3.0 \pm 0.3$ g; weights began to decrease by Day 7 and returned to prechallenge levels by Day 9 (Chart 1). In contrast, 4 of 22 (18%) of *C. parvum*-pretreated mice died from progressive L1210 growth (1, Day 5; 1, Day 7; and 2, Day 9), which is significantly greater mortality than in the controls ($p < 0.05$ by $\chi^2$ test). Moreover, the mean weight gain in this group was $6.5 \pm 0.6$ g and remained maximal through Day 9. In the surviving mice, weight loss and accompanying diminution of ascites was not observed until

![Table 1](image-url)
Day 11. By Day 13, all surviving C. parvum-pretreated mice appeared to be healthy and free of ascites; the small increase in weight above prechallenge levels at this time point probably represents normal weight gain associated with recovery from C. parvum toxicity (5).

**Effect of C. parvum on Cell-mediated Immunity.** Spleen cell-mediated cytotoxicity of mice pretreated with C. parvum (1.0 or 0.5 mg i.v.) was moderately but quite significantly suppressed compared with mice given L1210 cells without pretreatment (Table 2). The suppression became more apparent as the spleen cell to target cell ratio was lowered. Spleen cells from the few mice pretreated with these doses that did not display enhancement of tumor growth were nonetheless as suppressed as spleen cells from tumor-enhanced animals. While pretreatment with 0.25 mg of C. parvum i.v. did not cause significant suppression of cell-mediated cytotoxicity, several of these mice showed enhancement of their tumor. C. parvum pretreatment given i.p. produced significant suppression of cytotoxicity at the 0.5 but not at the 0.25-mg dose.

The spleen of mice pretreated with C. parvum was approximately 3 times the size of the spleen from mice given L1210 cells without pretreatment. Differential cell counts of Wright-stained spleen cell suspensions indicated that 80 to 90% of the cells in the spleen of control and C. parvum-pretreated mice were small lymphocytes and 3 to 4% were polymorphonuclear leukocytes. The C. parvum pretreatment suspensions contained about 1% large, immature lymphoid cells, and these were absent in suspensions from mice not receiving C. parvum. The major difference in the cellular composition of the spleens from the 2 groups of mice was the increased percentage of macrophages in the C. parvum-treated group, 15 to 20% versus 5 to 10% in immunized controls.

Because of the suggestion that C. parvum may activate macrophages that can suppress T-cell responses (19), it seemed pertinent to determine whether reducing the number of macrophages in the spleen suspensions could reverse or reduce the suppression of spleen cell-mediated cytotoxicity. Incubation of spleen suspensions on plastic dishes prior to mixing with target cells decreased the proportion of macrophages to 1 to 3% in both nonpretreatment and C. parvum pretreatment groups. However, this procedure did not effect any change in the degree of suppression of cell-mediated killing (Table 3).

**Effect of C. parvum on Cytotoxic Antibody.** Complement-dependent cytotoxic antibody was profoundly suppressed in mice pretreated with C. parvum i.v. at 1.0, 0.5, and 0.25-mg doses (Chart 2). In general, there was a close correlation between enhancement of leukemia L1210 and depression of

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**Table 2**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment (Day - 7)</th>
<th>% specific <strong>Cr release</strong> at spleen cell:target cell ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100:1</td>
<td>50:1</td>
</tr>
<tr>
<td>A</td>
<td>None (4)</td>
<td>59.3 ± 0.5**</td>
</tr>
<tr>
<td>C. parvum, 1.0 mg i.v. (5)</td>
<td>35.8 ± 5.3 (p &lt; 0.01)*</td>
<td>28.2 ± 5.6 (p &lt; 0.001)</td>
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<tr>
<td>B</td>
<td>None (4)</td>
<td>54.0 ± 0</td>
</tr>
<tr>
<td>C. parvum, 1.0 mg i.v. (5)</td>
<td>46.8 ± 1.2 (p &lt; 0.001)</td>
<td>47.8 ± 5.9</td>
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<tr>
<td>C</td>
<td>None (5)</td>
<td>56.2 ± 5.5</td>
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<tr>
<td>C. parvum, 0.5 mg i.v. (7)</td>
<td>38.3 ± 2.1 (p &lt; 0.02)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>None (3)</td>
<td>57.7 ± 4.9</td>
</tr>
<tr>
<td>C. parvum, 0.5 mg i.v. (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>None (8)</td>
<td>49.7 ± 2.2</td>
</tr>
<tr>
<td>C. parvum, 0.25 mg i.v. (9)</td>
<td>44.8 ± 2.8 (p &gt; 0.10)</td>
<td>19.2 ± 5.4 (p &gt; 0.20)</td>
</tr>
<tr>
<td>C. parvum, 0.10 mg i.v. (8)</td>
<td>51.7 ± 1.2 (p &gt; 0.20)</td>
<td>26.4 ± 3.9 (p &gt; 0.20)</td>
</tr>
</tbody>
</table>

**Notes:**
- All mice received 2.5 x 10⁶ L1210 cells on Day 0.
- Assay performed on Day 10; 5 x 10⁶ P815Y target cells.
- Numbers in parentheses, number of mice.
- Mean ± S.E.
- Compared with nonpretreatment group.
- Represents pool of 2 experiments.
Effect of removal of adherent cells on suppression of spleen cell-mediated cytotoxicity by C. parvum

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment (Day - 7)</th>
<th>Unfractioned spleen cells removed</th>
<th>Adherent cells removed</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None (2)</td>
<td>52.0 ± 5.0</td>
<td>53.0 ± 4.0</td>
<td>&gt;0.20</td>
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<tr>
<td></td>
<td>C. parvum, 1.0 mg i.v. (3)</td>
<td>32.7 ± 4.5</td>
<td>26.7 ± 4.1</td>
<td>&gt;0.20</td>
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<tr>
<td>B</td>
<td>None (2)</td>
<td>69.0 ± 5.0</td>
<td>70.5 ± 3.5</td>
<td>&gt;0.20</td>
</tr>
<tr>
<td></td>
<td>C. parvum, 0.5 mg i.v. (3)</td>
<td>35.0 ± 4.6</td>
<td>37.0 ± 5.7</td>
<td>&gt;0.20</td>
</tr>
</tbody>
</table>

* All mice received 2.5 × 10^6 L1210 cells on Day 0.
* Assay performed on Day 10; 5 × 10^6 P815Y target cells.
* Represents pool of 2 experiments; spleen cell to target cell ratio 25:1.
* Numbers in parentheses, number of samples, each of which represents 2 to 3 mice.
* Mean ± S.E.
* Unfractioned spleen compared with adherent cells removed.
* Spleen cell to target cell ratio, 100:1.

Table 3

The Correlation between the in vivo observations and the in vitro tests is important. Enhancement of tumor growth was always associated with profound suppression of cytotoxic antibody but was sometimes found with a normal degree of spleen cell-mediated cytotoxicity. Rejection of the tumor by C. parvum-pretreated mice was generally associated with higher, although suppressed, antibody levels and normal cell-mediated cytotoxicity. This was consistent with a previous study from our laboratory (S. J. Deegan and M. S. Mitchell, unpublished observations) that indicated that the growth of cytarabine-resistant leukemia L1210 could be enhanced by suppressing antibody synthesis with cytarabine, but not by inhibiting only cell-mediated immunity with allogeneic antilymphocyte serum.

Enhancement of leukemia L1210 by C. parvum resulted in the death of 4 of 22 (18.2%) of the mice given 10 × 10^6 tumor cells. In the remaining 18, the enhancement was temporary; although these animals appeared to be ill and had massive ascites, they eventually rejected the tumor and recovered. Preliminary data indicate that the regression of tumor in these mice was associated with recovery of cell-mediated and antibody-mediated cytotoxicity in vitro, but further studies are in progress.

DISCUSSION

Pretreatment of mice with C. parvum 7 days before challenge impaired their immunological response to the allogeneic leukemia L1210. This was demonstrated in vivo by enhancement of the growth of the tumor and in vitro by suppression of spleen cell-mediated and complement-dependent antibody-mediated cytotoxicity. The phenomenon was clearly related to dose, route of administration, and scheduling of C. parvum in relation to the time of tumor challenge. C. parvum given i.v. at 1.0-, 0.5-, or 0.25-mg doses produced enhancement of leukemia L1210 and suppression of antibody. In addition, the 2 higher doses caused suppression of spleen cell-mediated cytotoxicity. The lowest i.v. dose given, 0.1 mg, affected only cytotoxic antibody levels. C. parvum given i.p. produced enhancement and suppressed in vitro tests at a 0.5 but not at 0.25-mg dose. Although the importance of scheduling was not thoroughly investigated, C. parvum administration 7 days before tumor challenge caused enhancement, while treatment 1 day after tumor challenge had no effect. These dosage schedules have been used by other investigators who have reported antitumor effects of C. parvum in other systems (15, 22, 25).

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The only other documentation of enhancement of tumor growth by \textit{C. parvum} was by Mantovani \textit{et al.} (13), who noted that when leukemia L1210 was given i.p. to allogeneic C3H mice 9 days after 0.7 mg of \textit{C. parvum} i.v., 100% of the mice died as compared with only 60% of controls. This effect was highly schedule dependent in that \textit{C. parvum} pretreatment 4 or 5 days before tumor challenge resulted in increased protection against the tumor. These authors did not attempt to document an immunological basis for the enhancement.

We recognize that our tests principally measure responses to histocompatibility antigens in this allogeneic system. This system was chosen because it permitted a variety of simple in \textit{vitro} measurements of immunological reactions to the same strong immunogen, the tumor cell, and was not intended to serve as a model for demonstrating the \textit{in vivo} effects of \textit{C. parvum} on a syngeneic tumor. Nevertheless, the use of an allogeneic system made the enhancement in \textit{C. parvum}-treated mice all the more striking, since untreated mice easily rejected the tumor. Enhancement is more difficult to demonstrate in syngeneic systems where both enhanced and normal animals usually have progressively growing tumors.

The suppressive effects of \textit{C. parvum} on T-cell function have been well documented. Scott (18) demonstrated decreased ability of spleen cells from \textit{C. parvum}-treated mice to respond to phytohemagglutinin and mixed lymphocyte reactions and to act as effector cells in a graft versus host system. Castro (5) reported that \textit{C. parvum} markedly decreased thymic weight and reduced the percentage of \wedge antigen-positive cells in peripheral lymphoid organs; this was reflected by prolonged survival of skin allografts. Milas \textit{et al.} (14) confirmed many of these findings. Ruitenberg and Steerenberg (17) observed that rats pretreated with \textit{C. parvum} displayed impaired ability to expel \textit{Trichinella spiralis} from their intestinal tracts, which is a T-cell-dependent activity. Asherson and Allwood (1) noted impairment of the development of contact sensitivity to picryl chloride, and Scott (21) reported suppression of a delayed hypersensitivity response to sheep RBC in \textit{C. parvum}-treated mice.

Although \textit{C. parvum} has generally been observed to stimulate B-cell function (3, 9), suppression has also been reported. Warr and Sljivic (23) found that the generation of plaque-forming cells was impaired when \textit{C. parvum} was given before low doses of sheep RBC. They also found that the response to pneumococcal polysaccharide was suppressed when \textit{C. parvum} preceded the antigen by 1 or 7 days. Howard \textit{et al.} (9) reported that mice given \textit{C. parvum} and then irradiated and reconstituted with lymphoid cells produced fewer plaque-forming cells in response to pneumococcal polysaccharide than did comparable controls not pretreated with \textit{C. parvum}.

We have demonstrated here that \textit{C. parvum} induced suppression of both cellular and humoral immunity. Higher i.v. doses of \textit{C. parvum} (1.0 and 0.5 mg) suppressed spleen cell-mediated cytotoxicity; the effector cells in this short-term $^{51}$Cr assay have been shown to be cytotoxic T-cells (6). All i.v. doses from 0.1 to 1.0 inhibited cytotoxic antibody production, presumably a function of B-cells. It is conceivable, however, that suppression of a ‘helper’ T-cell could have been responsible for the impairment of humoral immunity.

Scott (19) reported that decreased T-cell function was mediated by activated macrophages elicited by \textit{C. parvum}. Removal of macrophages by adherence to glass beads or phagocytosis of iron particles restored T-cell responsiveness to phytohemagglutinin. Kirchner \textit{et al.} (11) similarly observed that the spleens of mice given \textit{C. parvum} i.p. contained macrophages that suppressed the responses of normal spleen cells to mitogens. We were unable to demonstrate reversal of suppression of T-cell cytotoxicity by removal of plastic-adherent cells. Although cells morphologically identifiable as macrophages were reduced to 1 to 3%, it is possible that the remaining few macrophages were sufficient to cause suppression. It is equally possible that suppressor macrophages had already acted \textit{in vivo} and that their removal \textit{in vitro} was insufficient to reverse the established T-cell suppression.

It is well recognized but worth restating that immunostimulants can be immunosuppressive. The lowest dose of \textit{C. parvum} that produced enhancement in mice, 0.25 mg, corresponds to a dose of about 65 mg for an average size human (37.5 mg/sq m $\times$ 1.7 sq m). This greatly exceeds the usual human dose of 5 to 10 mg. Nevertheless, we feel that these results make careful therapeutic design and monitoring imperative if this agent is to be used for immunophylaxis in man.

REFERENCES

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