Effect of Corynebacterium parvum on Colony-stimulating Factor and Granulocyte-Macrophage Colony Formation

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SUMMARY

Because Corynebacterium parvum has tumor-inhibitory properties and stimulates granulocyte-macrophage production, it may have clinical value in combination with chemotherapy. The leukopoietic effect of killed suspensions of C. parvum was studied in mice using the technique of in vitro clonal culture of hematopoietic cells. After C. parvum injection, there was a prompt, sustained elevation of serum colony-stimulating factor followed by an increase in granulocyte-macrophage precursor cells in the spleen and increases in blood mononuclear and granulocyte cells. Colony-stimulating factor production is suggested as a major mechanism of stimulation of granulocyte-macrophage proliferation by C. parvum. Since rapidly proliferating hematopoietic cells may have increased sensitivity to cytotoxic agents, the details of hematopoietic stimulation by C. parvum may be critical in the sequential timing of combined C. parvum and chemotherapy treatment to obtain maximal tumor inhibition and minimal hematopoietic toxicity.

INTRODUCTION

Killed suspensions of Corynebacterium parvum are capable of causing partial and complete tumor regressions (8, 9, 13, 34) and decreased metastatic spread (18, 21, 25) of certain experimental tumors. Preliminary results have suggested similar tumor-inhibitory effects in humans (17). It is believed that production of activated macrophages is an important component of C. parvum's antitumor activity. C. parvum-activated macrophages can inhibit tumor growth in vitro (12, 23), stimulate B-cell function (15, 16), and inhibit T-cell function (14). The mechanism by which C. parvum causes the generation of activated macrophages is not known, but systemic administration causes diffuse reticuloendothelial hyperplasia, and several studies have suggested the possibility that C. parvum may cause increased bone marrow macrophage production (5, 33).

This paper presents studies that detail a sequence of changes after C. parvum injection, including increase in CSF3 followed by increases in granulocyte-macrophage precursor cells and increases in peripheral blood mononuclear and granulocyte cells. These studies suggest that production of CSF may be a major mechanism of stimulation of granulocyte-macrophage proliferation by C. parvum. Precise knowledge of the kinetics of CSF elevation and the bone marrow response will probably be of importance if C. parvum therapy is to be most effectively and safely combined with cytotoxic chemotherapy in the treatment of cancer.

These studies utilized the technique of in vitro clonal culture of hematopoietic precursor cells of macrophages and granulocytes. CSF is a specific glycoprotein regulator required for granulocyte-macrophage colony proliferation in semisolid tissue culture media.

MATERIALS AND METHODS

Animals. The mice used in these studies were C57BL/6 males, 2 to 6 months old, from The Jackson Laboratory, Bar Harbor, Maine. In each experiment, only animals of similar age and size were used. WBC counts and differentials were obtained by nicking the tails of nonanesthetized mice. Sera were collected by bleeding from the retroorbital plexus under ether anesthesia and were stored at −20° before their assay in batches. Bone marrow cells were flushed from the femoral shaft into McCoy's medium and were pipetted to obtain single-cell suspensions. Bone marrow cells were counted in a hemocytometer.

C. parvum. Burroughs Wellcome C. parvum CN 6134N, Batch PX 398 (Burroughs Wellcome and Co., Research Triangle Park, N. C.), at 7 mg dry weight/ml suspension of washed, formalin-killed organisms in 0.9% NaCl solution containing 0.01% thioumeral, was used in these experiments. C. parvum was administered i.p.

Assay of CSF. A modification of the soft agar culture technique for hematopoietic cells (10) developed by Bradley and Metcalf (2) in 1966 was utilized. Cultures were plated in 35-mm Falcon plastic Petri dishes containing 1 ml of medium. As target cells for assay of CSF, C57BL/6 bone marrow cells from the pooled femoral marrows of 2 mice were added to give 50,000 cells/plate. Control and experimental mouse sera were assayed at doses of 0.01 and 0.02 ml added to each plate. The medium utilized was modified McCoy's medium (Grand Island Biological Co., Grand Island, N. Y.) to which 15% fetal calf serum (Grand Island

1 Supported in part by Grant CA12398 from the NIH.
2 To whom requests for reprints should be addressed.
3 The abbreviation used is: CSF, colony-stimulating factor.
Received February 23, 1976; accepted February 2, 1977.
Biological Co.) was added. Two thousand units each of penicillin and streptomycin mixture (Microbiological Associates, Bethesda, Md.) were added to each 100 ml of McCoy’s medium. Three % Difco Bacto Agar was boiled for 2 min, and, after being cooled to 40°, was added to the tissue culture medium at a ratio of 9:1 yielding a 0.3% agar concentration. This mixture was held at 37°, and the cells to be cultured were added. Plates were allowed to gel at room temperature for 20 min and then were incubated for 7 days at 37° in a humidified 10% CO₂-air atmosphere. The colonies were scored with a dissecting microscope at ×10, and aggregates containing 50 or more cells were scored as colonies. The above modifications of the soft agar culture technique have increased the sensitivity of the system to stimulation by CSF so that 0.01 to 0.02 ml of normal mouse serum added to the 1-ml culture is sufficient to routinely promote colony formation. These doses are in the range of linearity between the volume of added serum and the number of colonies developing. Without added serum, there is no colony formation.

**Assay of Colony-forming Cells.** The entire marrow contents of the shaft of 1 femur were expressed into 3 ml of McCoy’s Medium 5A, and pipetted repeatedly to obtain a single-cell suspension. Quantitative collection of femoral shaft marrow permitted calculation of colony-forming cells per femur. Aliquots sufficient to give 50,000 cells/ml were added to the culture medium with 0.025 ml/plate of pooled mouse serum known to have strong colony-stimulating activity. Quadruplicate cultures were plated, always including a control plate without CSF to check for spontaneous colony formation. Spleen cells were similarly cultured after single-cell suspensions had been obtained by teasing the spleen with sterile needles. Spleen cells were plated at a concentration of 100,000 cells a plate.

**Histology of Spleen and Liver.** Spleens and livers were examined histologically on Days 1, 3, 7, and 16 after the injection of *C. parvum*. Blocks from tissues fixed in 10% buffered formalin were embedded in paraffin, cut at 4 μm, and stained with hematoxylin and eosin.

**Cytology of Bone Marrow and Spleen.** Bone marrow and spleen smears were stained with Wright’s stain prior to differential counts. Technical problems made necessary the exclusion of many smears from analysis; therefore, the data in Tables 1 and 2 are limited.

**Statistical Analyses.** A 2-tailed Student’s t test was used for data analyses.

**RESULTS**

**Effect of Varying Doses of *C. parvum* on Bone Marrow Granulocyte-Macrophage Progenitor Cells.** Twenty-four hr after injection of *C. parvum* i.p., there was a depletion of total bone marrow cellularity which was dose dependent (Chart 1). There was no significant change in the incidence of granulocyte-macrophage progenitor cells per 50,000 bone marrow cells plated and a slight decrease in the number of granulocyte-macrophage progenitor cells per femur that was significant only for the 700-μg dose of *C. parvum*.

**WBC Response to *C. parvum*.** The dose of 1400 μg/mouse has been commonly used by other investigators to obtain tumor regression (8, 27). Therefore, this dose was used in the following experiments. The peripheral WBC response after i.p. injection of 1400 μg *C. parvum* was studied in 10 mice. By Day 7, the absolute numbers of granulocytes were 3 times higher in the *C. parvum* mice as compared to controls; monocytes were 5 times higher, and lymphocytes were significantly decreased (Chart 2).

**Sequential CSF and Granulocyte-Macrophage Progenitor Cell Response after Injection of *C. parvum*.** Groups of 10 mice received i.p. injections of 1400 μg of *C. parvum* and were assayed at intervals for serum CSF, bone marrow, and spleen colony-forming cells, as well as spleen and liver weight. At each time interval, a group of control mice treated with 0.9% NaCl solution was similarly assayed. Serum colony-stimulating activity levels were elevated 5-fold at 6 hr and decreased somewhat to lower but still elevated levels at Day 7, which persisted through Day 16 (Chart 3).

In order to better delineate the early changes in serum CSF after *C. parvum* treatment, groups of 10 mice as well as 0.9% NaCl-injected controls were studied at 1, 3, and 6 hr after i.p. injection of *C. parvum*. At 1 hr, a significant increase in serum colony-stimulating activity was detectable which rose progressively to 6 hr (Chart 4).

There was a prompt decrease in the number of bone marrow cells per femur to 75% of normal at 24 hr which persisted through Day 7 and had returned to normal at Day 16 (Chart 5). Colony-forming cells per femur were expressed as the percentage of normal colony formation to adjust for variation in culture conditions from run to run. There was a significant increase in the number of bone marrow colony-forming cells at Day 16.

Colony-forming cells in the spleen increased from unde

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![](chart.png)

**Chart 1.** Effect of *C. parvum* injection i.p. on incidence of in vitro granulocyte/macrophage colony-forming cells, cell counts of femoral bone marrows, and colony-forming cells per femur 24 hr after varying doses of *C. parvum*. Ten mice were in each group. All values are mean ± S.E. Significant differences from 0.9% NaCl solution control are indicated: *p < 0.05, **p < 0.01, ***p < 0.001.
detectable levels to an incidence of 39 ± 9 (S.E.) per 100,000 cells at 7-days and were again undetectable at 16 days (Chart 6). The increase in colony-forming cells in the spleen was accompanied by splenic enlargement; therefore, the absolute increase in colony-forming cells was much greater than the relative increases. There was a 2-fold hepatic enlargement at the 7- and 16-day intervals.

In no case was there in vitro colony growth of either bone marrow or spleen cells without a source of added CSF.

**Histology and Cytology of Spleen after Injection of C. parvum.** The spleens from mice treated with 1400 μg C. parvum i.p. were examined for histological changes 1, 3, 7, and 16 days after injection.

C. parvum injection produced striking hyperplasia of the cells in the red pulp of the spleen. Extramedullary hematopoiesis was markedly increased in comparison to control animals and involved erythroid, myeloid, and megakaryocytic cell lines. Increased numbers of macrophages were also seen in the red pulp on Days 1 and 3, with a decline in their numbers on Days 7 and 16. Extramedullary hematopoiesis peaked at Day 3 but continued to be increased, although declining at 16 days. Changes in the white pulp were also seen after the injection of C. parvum. The area occupied by the white pulp remained unchanged or was diminished. Germinal centers, a normal feature of mouse spleens, were absent within 24 hr after C. parvum administration. Only at 16 days were a few germinal centers again seen in treated animals. Three days after injection, the white pulp contained increased numbers of the immature lymphoid cells. The numbers of these cells reached a peak at 7 days and then diminished by Day 16.
precursor cells as well as mature myeloid and erythroid cells.

**Bone Marrow Cytology.** Bone marrow smears on Days 3, 7, and 16 showed striking myeloid hyperplasia with predominantly mature granulocytes (Table 2).

**Liver Histology after Injection of C. parvum.** Progressive liver enlargement occurred after C. parvum injection. At 24 hr, numerous thrombi were present in portal and central veins. In addition, frank centrilobular necrosis was seen in several sections. On Days 3, 7, and 16, liver sections showed perportal and focal intrahepatic inflammation. The inflammatory infiltrate consisted principally of mononuclear cells, but occasional neutrophiles were present in portal areas. These inflammatory foci frequently contained necrotic hepatocytes. By Day 16, inflammation was declining. Some control animals showed rare inflammatory foci in the hepatic parenchyma. Kupffer cells were also more prominent in animals receiving C. parvum at every time interval. Extramedullary hematopoiesis was not substantially increased morphologically, although some mouse livers did contain a few giant cells resembling megakaryocytes.

**In Vitro Effect of C. parvum on Colony Formation.** C. parvum was tested for the possibility that it might have the ability to stimulate colony formation when added directly in vitro to the plates. At doses from 0.138 to 70 µg/ml, there was no stimulatory effect, and doses greater than 0.55 µg were inhibitory to colony formation induced by mouse sera with a high titer of CSF. In addition, it was found that after a 2-hr incubation with 170- and 350-µg/ml doses of C. parvum, but not lower doses, colony formation of normal mouse cells was inhibited completely.

**DISCUSSION**

CSF has been shown to be a glycoprotein that is a specific regulator of granulocyte-macrophage proliferation and differentiation in vitro (28, 29). Many of its chemical characteristics are similar to those of erythropoietin, but the biological characteristics of the 2 poietins are quite distinct. To date, a preparation of CSF of sufficient purity to permit firm
C. parvum and Leukopoiesis

Table 1

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>No. of mice</th>
<th>% myeloid*</th>
<th>% erythroid*</th>
<th>% lymphoid*</th>
<th>No. of mice</th>
<th>% myeloid</th>
<th>% erythroid</th>
<th>% lymphoid</th>
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<tr>
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<td>8</td>
<td>12</td>
<td>80</td>
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<td>28.6 ± 3.9*</td>
<td>26.2 ± 5.1*</td>
<td>45.2 ± 8.8*</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>7.4 ± 1.5</td>
<td>12.0 ± 3.0</td>
<td>80.5 ± 4.0</td>
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* Values are mean or mean ± S.D.

Table 2

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<tr>
<th>Days after injection</th>
<th>No. of mice</th>
<th>% myeloid*</th>
<th>% erythroid</th>
<th>% lymphoid</th>
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<td>6</td>
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</table>

<table>
<thead>
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<th>C. parvum (1400 µg i.p.)</th>
<th>No. of mice</th>
<th>% myeloid</th>
<th>% erythroid</th>
<th>% lymphoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>82.2 ± 4.2*</td>
<td>13.4 ± 3.0*</td>
<td>4.3 ± 2.1*</td>
<td></td>
</tr>
</tbody>
</table>

* Values are mean or mean ± S.D.

establishment of its in vivo role in granulocyte-macrophage production has not been available, but there are considerable circumstantial data suggesting that CSF has a major physiological role in vivo (3, 20, 30).

The bulk of the evidence suggests that the colony-forming cell which is acted upon by CSF is a precursor cell committed to differentiation into the granulocyte-macrophage line and is not a pluripotent stem cell (1, 22). The progenitor cell responsive to CSF in granulocyte-macrophage production is probably analogous to the erythropoietin-sensitive cell in the erythroid cell line.

A wide variety of agents have previously been shown to be capable of elevating CSF including viral infections (10), endotoxin (24, 26), and antigenic stimulation (19). Most other agents cause a more transient elevation of CSF than the sustained elevation of CSF produced by C. parvum administration in these experiments.

Our findings vary from the statement of Dimitrov et al. (5) that C. parvum does not elevate serum CSF. Dimitrov et al., in their studies, routinely added 0.1 ml of mouse serum to each plate and obtained approximately 25 colonies with 4-hr post-C. parvum serum, whereas we have repeatedly found that 0.02 ml of 3-hr post-C. parvum serum is capable of inducing 80 colonies per 50,000 bone marrow cells plated. There may possibly be technical factors that account for the differences, e.g., Dimitrov et al. routinely dialyzed sera before testing, which we have not found necessary. Also, we have found in the past that, when some mouse sera in doses of 0.1 ml are added to 1-ml cultures, there may be an inhibition of colony formation which seems to be related to changes in the surface of the agar; some of these sera are highly active when added at lower doses.

These studies, in general, confirm the prior observations of Wolmark and Fisher (33) and Dimitrov et al. (5) that C. parvum can increase the incidence of granulocyte-macrophage colonies in the bone marrow. It is apparent, however, that when absolute numbers of bone marrow colony-forming cells are calculated, the increase in absolute numbers is not as great as suggested by the relative increases and that significant increases in granulocyte-macrophage colony-forming cells occurred only 16 days after a 1400-µg dose of C. parvum. There is, apparently, a differential depletion of non-colony-forming cells from the bone marrow after injection of C. parvum. The studies presented here have demonstrated a much more significant extramedullary increase, quantitatively, in colony-forming cells in the spleen.

It is postulated that CSF is an amplifying factor whereby precursor cells are stimulated to undergo more rapid proliferation to increase the number of differentiated cells. The present study suggests the hypothesis that C. parvum produced rapid and sustained elevations of CSF which in turn induced proliferation of the granulocyte-macrophage precursor cells. This resulted in an increase in production of circulating granulocytes and monocytes as well as the fixed macrophages which contributed to the splenic and hepatic enlargement. Although the marked increases in the number

MAY 1977

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of peripheral blood granulocytes and monocytes as well as the increased numbers of myeloid cells in the spleen strongly suggest increased production, this is only presumptive, since no kinetic studies were done to prove this point. Doubtless, the effect of C. parvum is complex, and there may be other physiological mechanisms for its action on hematopoiesis, but it is proposed that elevation of CSF by C. parvum may be an important mediator of the granulocyte-macrophage proliferation induced by C. parvum.

These studies have not established whether the elevation of CSF after C. parvum injection is a direct or indirect effect. Since the liver is a rich source of CSF, it is possible that the liver changes caused by the C. parvum lead to release of CSF in a nonspecific manner. In studies by Chan (4), destruction of most of the liver by carbon tetrachloride was followed in some cases by moderate rises in serum CSF levels. We have previously found that partial hepatectomy in mice did not cause consistent elevations in serum CSF (R. S. Foster and D. Metcalf, unpublished observation). Also, in humans with advanced cirrhosis, some, but not the majority, were found to have elevated serum CSF levels in the absence of intercurrent infections (11). Thus, although a number of changes in hepatic function are accompanied by moderate changes in serum CSF, the serum CSF elevations that we have found after C. parvum treatment are unusual in that the elevations are particularly high and are sustained for many days.

As a potential therapeutic agent for treatment of patients with malignant neoplasm, C. parvum has most intriguing possibilities because of its ability to inhibit tumor growth as well as stimulate certain aspects of myelopoiesis. The possibility that C. parvum might be used in combination with chemotherapy and radiotherapy to prevent or reverse myelosuppression has been suggested (5). It should be pointed out, however, that rapidly proliferating hematopoietic cells may have increased sensitivity to cytotoxic agents (6, 7, 31, 32). The present studies have suggested a very rapid stimulation of myelopoiesis after high doses of C. parvum, and it should be considered that the sequential timing of cytotoxic chemotherapy and C. parvum treatment may be critical to obtain maximal tumor inhibition as well as minimal hematopoietic toxicity. We have recently completed preliminary studies which have demonstrated an increased lethality in mice when C. parvum is given 24 hr before a cycle-active chemotherapeutic agent, 5-fluorouracil. There is also some evidence for protection from hematopoietic toxicity of 5-fluorouracil when C. parvum is given 24 hr after the cytotoxic agent.

REFERENCES


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