Synergistic Effect between Neutrophils and Corynebacterium parvum in the Process of Macrophage Activation

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ABSTRACT

Adoptive transfer of granulocytes exposed to Corynebacterium parvum in vivo or in vitro efficiently activates murine peritoneal macrophages in vivo. A comparison of the amount of bacteria required to produce similar levels of cytolytic activity indicated that 2 x 10^7 intracellular (neutrophil) bacteria were as efficient as 1 x 10^9 bacteria introduced directly.

The time courses of macrophage cytotoxicity induced by these two methods were found comparable. Similar levels of activity were also observed in tumor cytostasis and inhibition of phytohemagglutinin-induced mitogenesis assays. However, when spleen size or cell division in the spleen or bone marrow was assessed, differences were found. Direct C. parvum injection caused marked splenomegaly. The proliferative fraction of cells in the spleen and bone marrow was significantly increased over both control and C. parvum-induced neutrophil groups.

These results further document the nature and efficiency of the interaction between neutrophils and bacteria in macrophage activity. The findings are significant in that they demonstrate a novel way to activate macrophages in vivo without causing the potentially harmful side effects which result from direct injection of the biological response modifier.

INTRODUCTION

Numerous studies have established the importance of granulocytes in the efferent arm of the host response against bacteria (5, 13). More recently, our group and others have demonstrated a role for these cells in the afferent arm of the immune response. Transfer of antigen-induced neutrophils to normal mice has been shown to stimulate the tumoricidal activity of macrophages and mitogen responses of lymphocytes (2, 9, 12).

Our observations suggest that granulocytes play an important role in the activation of peritoneal macrophages after administration of Corynebacterium parvum. We have shown that the bacteria are initially ingested by neutrophils which in turn are phagocytosed by macrophages. Furthermore, we demonstrated that only those macrophages which contain intracellular C. parvum are cytotoxic at the peak period of reactivity (Day 4) (1). In our laboratory, granulocytes isolated early in the response (5 hr) are highly efficient at activating murine macrophages in normal recipients to similar cytolytic levels as are achieved with direct injection of bacteria (2).

These results stimulated further investigation of the exact role neutrophils and bacteria play in the induction of macrophage tumoricidal activity. Furthermore, it was important to determine if the time course of the response was similar with the 2 different regimens and if the nonspecific side effects of the response to C. parvum were equal. The results in this paper demonstrate a way to reduce the amount of C. parvum needed to induce tumoricidal macrophages, while avoiding secondary, clinical complications.

MATERIALS AND METHODS

Animals. A x C57BL/6 F1 mice, 6 to 8 weeks olds, were obtained from the Trudeau Institute (Saranac Lake, N. Y.).

Cell Cultures. H-S3 cells were used as target cells in the macrophage cytotoxicity assay. Cells were grown in minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with L-glutamine, gentamycin (20 μg/ml), and 10% fetal bovine serum (KBC Biological Co., Kansas City, Mo.).

Target Cell Preparation. H-S3 cells were seeded into 25×2 cm flasks 2 to 3 days before use. Before the assay, medium was poured off, and fresh medium containing 5 μCi of [3H]thymidine (Schwarz/Mann, Spring Valley, N. Y.) per ml was added. The cells were then incubated 24 to 30 hr. Radiolabeled cells were dispersed from the tissue culture flask by washing the cells from the plastic with strong jets of medium from a Pasteur pipet. Cells were washed, counted, and adjusted to a concentration of 5 x 10^5 viable cells per ml. Target cell viability was always greater than 95% after dispersion. One x 10^6 target cells per well were used in each macrophage cytotoxicity assay.

Peritoneal Exudate Cells. The peritoneal exudate cells were harvested from mice killed by cervical dislocation. Cells were obtained by washing the peritoneal cavity with 10 to 20 ml of ice-cold PBS and were washed in minimal essential medium.

Neutrophil Harvest. Neutrophils were recovered as above at 5 hr after i.p. immunization with C. parvum. The peritoneal exudate cells were layered on Ficoll/Hypaque and centrifuged at 400 × g for 20 min. Neutrophils were recovered from the pellet of the Ficoll/Hypaque gradient. Cell recovery averaged 2 to 3 x 10^6 neutrophils per mouse killed. Greater than 90% of the cells were morphologically identified as granulocytes.

Macrophage Harvest. Macrophages were recovered from the peritoneal exudate cells as described above, 4 days after i.p. injection of C. parvum or C. parvum-containing neutrophils. The percentage of macrophages in the peritoneal exudate cells was determined by differential staining using Wright's stain. The peritoneal exudate cells were counted in a hemocytometer and were diluted to a concentration of 2.0 x 10^6 macrophages per ml to be used in a cytotoxicity assay.

Adherent Macrophage Cytotoxicity Assay. The macrophage cytotoxicity assay was performed in 96-well microtiter plates using [3H]thymidine-prelabeled targets. Macrophages were added to microtiter...
plate wells in a volume of 0.1 ml at a concentration of 20 × 10^6 macrophages per ml. Cells were allowed to adhere for 1 to 2 hr, and wells were washed vigorously to leave only adherent cells (>85% macrophages). Following washing, target cells were added to wells in a volume of 0.2 ml at a concentration of 5 × 10^6 cells per ml. The assay plates were incubated 18 to 20 hr at 37°C in a humidified CO_2 incubation.

After incubation, the percentage of specific ³H release was calculated by removing 0.1 ml of supernatant from each well and counting the amount of activity in a scintillation counter (Packard Instrument Co., Rockville, Md.). Maximum release was determined by placing 1 × 10^6 cells directly into Aquasol and counting. Spontaneous release was determined by incubating target cells in medium for the duration of the assay and ranged between 7 and 15% for these experiments.

The percentage of specific ³H release for each sample was calculated as follows.

\[
\text{% of specific release} = \frac{\% \text{ of release with macrophages} - \% \text{ spontaneous release}}{\% \text{ maximum release} - \% \text{ spontaneous release}} \times 100
\]

**Cytostasis Assay.** One × 10^6 unlabeled H-S3 cells were added to adherent macrophage effector cells in flat-bottomed 96-well tissue culture plates. After a 24-hr incubation, 0.5 μCi of [³H]thymidine was added to each well. The assay was harvested on an automated cell harvester 6 hr later. Cytostasis was determined as follows.

\[
\text{% of cytostasis} = \frac{\text{cpm of target cells macrophages} - \text{cpm of macrophages alone}}{\text{cpm of target cells alone}} \times 100
\]

This laboratory has found previously a lack of cold thymidine artifacts using this assay with C. parvum-activated macrophages by comparing it to flow cytometric analysis of growth inhibition (7).

**Inhibition of PHA-pulsed Spleen Cell Mitogenesis.** Spleen cells were aseptically removed from normal mice. Spleens were disrupted by expression through Nitex fabric. Cells were washed and centrifuged on Ficoll-Hypaque, and mononuclear cells were adhered twice on tissue culture plastic. Nonadherent cells (>98%) lymphocytes were then added to macrophage monolayers that had been prepared as described above for cytotoxicity assays, or they were added to microtiter plate wells without macrophages. Lymphocytes ± macrophages were pulsed with PHA (0.5 μg/well) for 24 hr. Then, 0.5 μCi of [³H]thymidine was added to each well for 6 hr to assess mitogenesis or inhibition of mitogenesis.

**Macrophage Activation.** Eight- to 12-week-old animals were used in all our studies. C. parvum (Burroughs Wellcome Co., Research Triangle Park, N. C.) at a concentration of 700 μg/0.1 ml was injected i.p. to activate macrophages or to induce neutrophils used for transfer studies. Neutrophils were harvested and purified as described above. Two to 5 × 10^6 neutrophils were resuspended in 0.5 to 1.0 ml of PBS and injected into mice i.p.

**Disruption of Neutrophils.** Neutrophils once purified and resuspended as usual were subjected to repeated freezing and thawing. Cells were examined for viability until no viable cells were found (3 cycles). Alternatively, neutrophils were disrupted by sonicating for 2.5 min (5 cycles at 30 sec each). Either of these materials was injected i.p., and macrophages were tested as described for cytotoxicity.

**Cytotoxicity Analysis.** The identification of bacteria-containing cells was done by cytotoxicity analysis. Cytocentrifuge slide preparations were stained with Wright's stain to identify cell morphology and cells with intracellular bacteria. A minimum of 100 cells per slide was identified for morphology and/or presence of C. parvum.

**In Vitro Incubation of Human Peripheral Blood Neutrophils with C. parvum.** Human peripheral blood neutrophils were obtained as follows. Blood was collected in a sterile, heparinized syringe, diluted 1:3 in RPMI, and layered in Ficoll-Hypaque. The gradients were centrifuged at 400 × g for 20 min, and the cells in the pellet fraction were collected. These cells were added to 3% dextran in 1:1 volume ratios and allowed to settle at 1 x g for 1 hr. The cells in the upper portion of the gradient were collected and pelleted. Fifteen ml of sterile H_2O were added for 30 sec, quickly followed by 35 ml of RPMI (with 10% fetal bovine serum). Neutrophils (>90%) of cells were washed once, counted, and seeded into Costar plate wells at a concentration of 3 × 10^6 cells per well. C. parvum was added to the neutrophils at a concentration of 27 μg per well. Neutrophils were incubated with or without C. parvum for 3 hr at 37°C and then recovered by washing wells with jets of medium from a Pasteur pipet and washed in RPMI at 200 × 3 times. Free bacteria were not pelleted using this g force while cells were recoverable. After 3 washes, cells were counted, tested for viability (trypan blue exclusion), and suspended in PBS at a concentration of 5 × 10^6 neutrophils per ml. One ml of this cell suspension was injected per mouse i.p.

**Cell Cycle Analysis.** Flow cytometric analysis was carried out as described previously (7). Briefly, an Ortho ICP-22 flow cytometer was used for all measurements. Single-parameter DNA profiles were stored directly in a Radio Shack (Fl. Worth, Texas) TRS-80 Model I microcomputer interfaced to the ICP-22. Software was created which permitted histogram storage and cell cycle analysis (modified from the method of Dean and Jett (4)). DNA quantitation was carried out in propidium iodide (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in PBS containing 1% type 1A RNase (Sigma) made up to 0.2% in Triton X. Cells were analyzed after a 5-min incubation.

**RESULTS**

**Determination of the Dose of the C. parvum or C. parvum-induced Neutrophils Needed to Activate Macrophages.** To determine what role the neutrophils and C. parvum play in macrophage activation, we first compared the doses of C. parvum or C. parvum-induced neutrophils needed to elicit cytotoxic macrophages. Chart 1A shows that 90-, 175-, and 350-μg doses of C. parvum did not enhance macrophage cytotoxicity over unstimulated levels. A dose of 700 μg, however, enhanced cytotoxicity significantly. When C. parvum-induced neutrophils were used to elicit cytotoxic macrophages, we found less than 1 × 10^6 neutrophils were needed to induce macrophages with significant cytotoxic activity (see Chart 1B). Furthermore, macrophages from animals receiving 5 × 10^6 neutrophils or 700 μg of C. parvum were found to have the same cytotoxic activity.

The data in Chart 1 can also be used to compare the number of bacteria injected in each group. Significant cytolytic activity was induced with as few as the 2 × 10^7 bacteria present in 1 × 10^6 neutrophils. This compares to a dose of 1 × 10^10 bacteria required to induce comparable cytotoxic levels in the absence of granulocytes.

![Chart 1](chart.png)

Comparison of the Time Course of Activation and Spleen and Bone Marrow Proliferation by the 2 Stimulation Procedures. We investigated how the 2 different macrophage activation procedures affected cytotoxic macrophage production, spleen size, and the percentage of cells undergoing cell division in the spleen and bone marrow. Chart 2C shows that the kinetics of macrophage cytotoxicity was similar when both types of macrophage activators were used (700 μg of C. parvum versus 5 × 10^6 neutrophils containing 2 × 10^7 bacteria).

In contrast, 700 μg of C. parvum induced a marked splenomegaly, whereas spleen size was not affected when C. parvum-induced neutrophils were used (Chart 2B). The proliferative fraction of cells in the spleen was also higher following direct C. parvum injection (Chart 2A). A burst of bone marrow proliferation was detected 2 days after direct C. parvum injection (Chart 2D). This was followed by a return to approximately normal levels of cell division by 4 days. In contrast, mice receiving C. parvum-induced neutrophils did not have significantly higher levels of cell division than seen in normal bone marrow.

Comparison of Cytotoxicity, Cytostasis, and Suppression of PHA-pulsed Lymphocyte Mitogenesis by C. parvum-induced Macrophages. Using both methods of macrophage activation, we investigated macrophage activity assessed in lymphocyte blastogenesis, tumor cell cytostasis, and cytotoxicity assays (Chart 3). The results indicated that cytostatic, cytolytic, and suppressor cell activities were similar with both activation procedures.

**Activation of Macrophages by Use of Neutrophils Exposed to C. parvum in Vivo.** The above results suggested that neutrophils may contribute to the immunostimulatory activity of C. parvum. That is, by localizing C. parvum intracellularly within a neutrophil, amounts of C. parvum that do not stimulate tumoricidal macrophages when injected directly can cause neutrophils to activate or contribute to macrophage activation.

To further investigate the apparent synergistic effect of C. parvum and neutrophils, human peripheral blood neutrophils were incubated in vitro with or without C. parvum (see "Materials and Methods").

**Table 1.**

<table>
<thead>
<tr>
<th>Macrophage activator</th>
<th>% of specific (^{3}H) release</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>3.7 ± 0.4^a</td>
</tr>
<tr>
<td>C. parvum*</td>
<td>20.3 ± 1.0</td>
</tr>
<tr>
<td>Peripheral blood neutrophils</td>
<td>8.5 ± 1.0</td>
</tr>
<tr>
<td>Peripheral blood neutrophils + C. parvum*</td>
<td>23.3 ± 1.8</td>
</tr>
</tbody>
</table>

^a Mean ± S.D. of triplicate assays from a representative experiment.

^b Murine peritoneal macrophages induced by 700 μg of C. parvum injected i.p.

^c Human peripheral blood neutrophils (5 × 10^6) incubated for 3 hr in vitro at 37°C with or without C. parvum before i.p. injection.

We found that human peripheral blood neutrophils ingested C. parvum effectively and were easily recovered. These cells were used in an attempt to activate murine macrophages as carried out previously with the C. parvum-elicited mouse peritoneal neutrophils. While normal peripheral blood neutrophils did not induce cytotoxic murine macrophages, human blood neutrophils containing bacteria were able to activate tumoricidal macrophages (Table 1). The levels of activity were similar to those seen previously with the murine granulocytes (2).

**DISCUSSION**

Stimulation of mice i.p. with C. parvum results in a series of distinct inflammatory changes which lead to the activation of cytolytic macrophages (1, 2). These steps were characterized by: neutrophil ingestion of C. parvum (first 5 hr); macrophage phagocytosis of C. parvum-containing neutrophils (>5, <40 hr); and subsequent appearance of macrophages with intracellular bacteria (>40 hr). This change of the cellular host of the bacteria and the predominant cell types found at the inflammatory site suggested a close interaction between the neutrophil and the macrophage and a stepwise mechanism by which neutrophils contribute to macrophage activation. The importance of intracellular C. parvum in the macrophages of Days 3 and 4 was emphasized by the finding that cytolytic activity was associated predominantly with macrophages containing bacteria (1).

We have reported previously that viable, intact neutrophils are unnecessary for macrophage activation (2). Since released bacteria could be the active component of C. parvum-induced neutrophils, it was therefore of interest to investigate the equivalent dose of bacteria that neutrophil-recipient mice were receiving in our experiments. We found that 1 × 10^10 bacteria had to be injected directly to optimally stimulate cytotoxic macrophages.
When bacteria-containing neutrophils were transferred to recipients, only 2 x 10^7 bacteria were introduced to the host. This number of bacteria is approximately 500 times lower than the dose needed to induce cytotoxic macrophages when injected directly. Since normal neutrophils or neutrophils exposed to nonstimulatory pyridine extracts of C. parvum (2) also did not induce cytotoxic macrophages, there appears to be a cooperative effect between the bacteria and the neutrophils in the induction of cytotoxic macrophages. The granulocytes may be serving as efficient vehicles for targeting the bacteria to the macrophages or as a source of modified bacteria with enhanced stimulating activity. One other group has described a synergistic effect between antigen and neutrophils in the regulation of immune cells (10, 15). Their reports describe a neutrophil-derived factor that acts with antigen to enhance T-cell DNA synthesis.

We found that the time course of macrophage cytotoxicity stimulated by C. parvum-induced neutrophils was very similar to that of macrophages from mice given C. parvum directly. Our previous studies have indicated that, when direct C. parvum injections are used to activate macrophages in vivo, the characteristics and cytotoxic potentials of the cells change with time. The in vivo mechanisms which control these time-dependent macrophage subpopulations are still undefined. However, it does not appear that using C. parvum-induced neutrophils to activate macrophages disrupts these regulatory mechanisms. This is further evidenced by the data showing that cytostatic and suppressor macrophages are induced by C. parvum-induced neutrophils as well as by direct C. parvum administration.

The nonspecific hematological and lymphoid proliferation which is so detrimental to combined chemoimmunotherapy procedures is absent using the C. parvum-granulocyte activation procedure. Both stimulation procedures induced macrophage populations with similar cytotoxic potentials. Whereas spleen size remained virtually unchanged in animals receiving C. parvum-induced neutrophils, the direct C. parvum recipients exhibited marked splenomegaly. A similar pattern was seen in the number of cells undergoing cell division in the spleen and bone marrow. Suggestions that determinants of C. parvum that cause splenomegaly are present on the bacterial surface and are removed with chemical treatment (3) might too indicate that neutrophils have altered the bacteria. These results are of considerable interest with respect to the use of C. parvum in nonspecific immunotherapy in association with chemotherapy.

Data presented here suggest that the reticuloendothelial acti-

\[\text{vating potential of } C. \text{parvum may be tapped without causing secondary clinical effects (8, 11) by introducing } C. \text{parvum in neutrophils. Many studies are now under way to separate the reticuloendothelial activating component(s) of } C. \text{parvum and to eliminate clinical side effects (3, 6, 14). The model described here may provide an attractive alternate therapeutic approach.}

\text{ACKNOWLEDGMENTS}

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\text{REFERENCES}

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