Effect of Glucan on Granulopoiesis and Macrophage Genesis in Mice

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SUMMARY

Glucan, a potent reticuloendothelial stimulant, is a glucopyranose polysaccharide derived from zymosan. Because of glucan's potential as an immunotherapeutic agent, we performed studies in order to determine its effect on granulopoiesis and macrophage production in mice. One week after the i.p. injection of 4 mg of glucan, there was a tenfold increase in colony-forming cells in the spleen and approximately a twofold increment of cells in the bone marrow and the peritoneal cavity capable of colony formation in vitro. There was a relative and absolute increase in the number of pure macrophage colonies from bone marrow and spleen. The total macrophage content in spleen, peritoneal cavity, and bone marrow was also increased in the treated mice. Serum from glucan-injected mice had high colony-stimulating activity levels, and the peritoneal macrophages elaborated increased colony-stimulating activity in vitro as compared to controls. Peripheral white blood cell counts were two times greater than those of control in the glucan-treated mice. These studies indicate that glucan administration results in increased granulocyte and macrophage production. The enhanced leukopoiesis is probably mediated in part by augmented release of colony-stimulating activity from macrophages. These observations suggest that the use of glucan as an immunotherapeutic agent can result in an increased number of available effector cells.

INTRODUCTION

A number of agents referred to as reticuloendothelial stimulants or macrophage activators have been considered useful in immunotherapy (1, 13, 15, 20, 27, 28). These agents are frequently of plant or microbial origin and have a multitude of biological effects. The immunopharmacology of the macrophage activators has recently come under close scrutiny because of their potential role in cancer therapy.

Pillemer and Ecker (30) applied the name zymosan to a yeast cell wall fraction of Saccharomyces cerevisiae which produced prominent hyperplasia and hyperfunction of the reticuloendothelial system when administered to experimental animals. Glucan, a polysaccharide of approximately 6500 daltons, has been isolated from zymosan and shown to be responsible for most of the stimulatory effects on the reticuloendothelial system (6, 31). Glucan consists of a chain of glucopyranose units joined by 1-3 glucoside linkages. When glucan is injected into rodents, it causes a marked increase in size and weight of the spleen, liver, and lungs, and appears to increase macrophage activity in these organs (7-9). Glucan administration has been reported to cause transient tumor regression in rats with Shay chloroleukemia (8) as well as inhibition of tumor growth in syngeneic systems such as B16 mouse melanoma and BW 10232 adenocarcinoma (24). There is also some limited experience with glucan immunotherapy in man (24, 25).

We undertook a series of experiments designed to assess the effect of glucan on granulocyte-monocyte progenitor cells and macrophage genesis in the mouse.

MATERIALS AND METHODS

Glucan was prepared in the laboratory of Dr. N. Di Luzio by techniques previously described (19). The material was provided in a stock solution of 37 mg/ml and diluted to 2 mg/ml in 5% dextrose. The preparation contained no endotoxin as determined by the limulus assay.

Female DBA/2 mice, 8 to 10 weeks old, were used in all experiments. The mice received i.p. injections of 2 mg glucan (approximately 200 mg/kg) on Days 0 and 1 and were sacrificed by cervical dislocation 6, 10, 15, and 20 days after the 1st injection. Control mice received equal volumes of 0.9% NaCl solution or 5% dextrose. A minimum of 3 mice/group were used in each experiment. The spleens of glucan-treated mice were removed aseptically, and single-cell suspensions were prepared in a medium (Flow Laboratories, Inglewood, Calif.) with 15% fetal calf serum and antibiotics. Bone marrow was flushed from the femora, and the cells were suspended in the same medium. Peritoneal cells were obtained by washing the peritoneal cavity with Hanks' balanced salt solution containing 10 units of preservative-free heparin per ml, 15% fetal calf serum, and antibiotics. The cells were washed twice and resuspended in medium. Total nucleated cell counts in the spleen, femur, and peritoneal cavity were determined by using a hemocytometer. The number of macrophages present in bone marrow, spleen, and the peritoneal cavity was determined by differential counts performed on cytocentrifuge preparations stained with Giemsa and for α-naphthyl butyrase (32).

Cells from the 3 sources were assayed for colony-forming capacity in agar. A single-layer culture method was used with 0.3% agar, α medium, and 15% fetal calf serum using techniques previously described (16, 18). An extract of 1 Supported by USPHS Grants CA 15688 and CA 15619.
2 To whom requests for reprints should be addressed.
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JUNE 1977 1739
pregnant mouse uterus was used as the source of CSA (3). This preparation had an activity of 3000 to 6000 colonies/mg protein. Fifty µl were added to each culture, providing a CSA concentration known to stimulate maximal colony formation. Plates were incubated in a humidified environment of 7.5% CO₂ in air at 37°C, and colonies were enumerated after 7 to 10 days for bone marrow and spleen cultures and 30 days for peritoneal cell cultures. The minimum colony size was taken as 50 cells. The ability of spleen cells to clone without added CSA ("autostimulation") was studied by plating high concentrations of cells (1 × 10⁶ to 4 × 10⁶/plate), and the colonies and clusters were counted at Day 7. Colonies were examined morphologically at high power with an inverted microscope, and selected colonies were picked with a finely drawn pipet, washed in phosphate-buffered saline (No. 420; Grand Island Biological Co., Grand Island, N.Y.), and deposited on glass slides with a cytocentrifuge for morphological and cytochemical analysis.

In order to assay serum CSA activity, 0.1 ml of serum was added to normal mouse bone marrow cultures in the double-layer agar culture system as previously described (16, 17). Glucan-stimulated and control macrophages (1 and 2 × 10⁵/plate) were also incorporated as feeder layers in 0.5% agar underlayers to test for the elaboration of CSA in the double-layer system.

Blood was obtained by cardiac puncture in lightly etherized mice, and WBC and differential counts were performed.

RESULTS

Glucan administration had relatively little effect on total nucleated cell counts in spleen and femur, but the CFU-C per spleen was increased tenfold in the glucan-treated animals 7 days after injection when compared to controls (Table 1). Bone marrow CFU-C were increased approximately 75% in the treated group (P ≤ 0.05; t test) (Table 1). These studies on spleen and bone marrow were repeated 10, 15, and 20 days after injection. By 20 days there was a further increase in spleen and bone marrow CFU-C (Table 1).

Gluca injection produced a consistent increase in the total number of cells recovered from the peritoneal cavity. The number of macrophages and macrophage colony-forming cells in the peritoneal cavity were 2 times higher in the treated group (Table 1).

Spleen cells from glucan-treated animals formed colonies without added CSA more readily than did spleen cells from controls (Table 2). At a concentration of 4 × 10⁶ cells/plate, there were 4 times more autostimulated CFU-C, suggesting increased numbers and/or activity of colony-stimulating cells. Normal spleen and bone marrow CFU-C were of granulocytic, monocyte-macrophage, and mixed types. In cultures from glucan-treated animals, 40% of the colonies were exclusively macrophage (attaching to and spread on the

### Table 1

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<thead>
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<th></th>
<th>Spleen*</th>
<th>Bone marrow*</th>
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<tr>
<td></td>
<td>Viable cell count (× 10⁸)</td>
<td>Total CFU-C (× 10³)</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 20</td>
</tr>
<tr>
<td>Glucan</td>
<td>1.3 ± 1</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 ± 2</td>
<td>1.4 ± 1</td>
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### Table 2

<table>
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<tr>
<th></th>
<th>Peritoneum*</th>
<th>Peripheral blood leukocytes*</th>
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<tbody>
<tr>
<td></td>
<td>Peritoneal cells (× 10⁶)</td>
<td>Total macrophages (× 10⁶)</td>
</tr>
<tr>
<td>Glucan</td>
<td>7.3 ± 0.7</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.2</td>
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* Seven days after glucan injection.

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bottom of the plate), whereas these constituted only 2 to 3% of the colonies in control cultures. The colonies formed from peritoneal cells were all of the macrophage type. Total macrophage counts in spleen, peritoneal cavity, and bone marrow were increased 2 to 3 times in the treated mice.

Serum from glucan-treated mice had increased CSA in comparison with control (Table 2). Glucan-activated macrophage underlayers also exhibited markedly increased CSA (Table 2). Peripheral WBC counts were 2 times greater than control values in glucan-treated mice (Table 1). There was no significant difference in the differential counts.

**DISCUSSION**

A variety of chemicals and biological materials are referred to as macrophage activators (1, 4, 15, 27, 28). These substances cause a characteristic general increase in macrophage function (5). More importantly, some are reported to induce the capacity for syngeneic tumor cell cytotoxicity (1, 15, 21, 29). However, all "activated" macrophages may not exhibit cytotoxicity. There is now a large literature on macrophage activation and the role of macrophages in containing and combatting tumor growth (11–13, 20, 28). A lymphocyte product (macrophage-activating factor), released during mitogen or antigen stimulation, is also a prominent macrophage activator (5, 12). Nonspecific immunotherapy is believed to be mediated in animals by the direct and indirect activation of macrophages. These considerations constitute much of the rationale for trials of nonspecific immunotherapy in man.

Macrophage tumor cell destruction may relate to the numbers of effector cells present at the site of tumor growth (2, 10, 11) as well as to the capacity for syngeneic cytotoxicity. In this regard, *Corynebacterium parvum* and *Bacillus Calmette-Guérin* have both been shown to increase the number of macrophages present in certain organs, as well as the number of granulocyte-monocyte precursor cells capable of colony formation in vitro (CFU-C) (14, 33, 34).

Glucan, a partially purified product of zymosan, is a potent reticuloendothelial stimulant and appears to activate macrophages. Glucan is also reported to have in vivo antitumor activity in several animal systems (24). We undertook to assess the effect of this potential immunotherapeutic agent on granulocyte and macrophage production in the mouse. The i.p. injection of glucan led to increased numbers of peritoneal exudate cells and to a prominent increase in the size of the liver and spleen. The number of colony-forming cells in the spleen increased by tenfold 1 week after glucan injection, and there was a small but statistically significant increase in bone marrow CFU-C. In addition to a net increase in granulocyte-monocyte precursor cells, there was an apparent change in the major direction of differentiation with a relative and absolute increase in pure macrophage colonies. Cells in the peritoneal cavity of mice have recently been shown to be capable of exclusively macrophage-type colony formation in agar under the influence of CSA (22, 23). These macrophage colony-forming cells were increased twofold over control in glucan-treated animals. The augmented production of macrophages in vitro was found to coincide with in vivo events. Total macrophage counts in spleen, peritoneal cavity, and bone marrow were substantially increased in mice receiving glucan.

Studies of CSA production in vivo and in vitro were conducted in an effort to define the mechanism of increased leukopoiesis. Colony and cluster formation occurred in agar cultures of spleen cells from treated animals plated at high density without an exogenous source of CSA. This phenomenon of autostimulation is due to increased numbers or activity of colony-stimulating cells (monocytes and macrophages) capable of releasing CSA in the culture dish (18, 26). Furthermore, glucan-activated macrophages elaborated considerably more CSA than did control cells when tested as a feeder layer in the agar culture system. The serum from glucan-treated mice also had increased CSA. Supporting evidence that the increased CSA resulted in augmented leukopoiesis in vivo was obtained with the observation of twofold higher peripheral WBC counts in the treated mice.

Our data indicate that glucan administration increased granulocyte and monocyte-macrophage production in mice. The augmented leukopoiesis is likely mediated by increased release of CSA from macrophages occurring concomitantly with the activation process. These observations suggest that when glucan is used as an immunotherapeutic agent it can increase the number of available effector cells.

**REFERENCES**

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