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COMPARISON OF GLUCAN AND MURAMYL DIPEPTIDE IN STIMULATION OF PARTICLE CLEARANCE AND HOST DEFENSE IN MICE

presented by

Dirk Warren Sprenger

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Martha Thomas
Major professor

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COMPARISON OF GLUCAN AND MURAMYL DIPEPTIDE IN STIMULATION OF PARTICLE CLEARANCE AND HOST DEFENSE IN MICE

By

Dirk Warren Sprenger

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ABSTRACT

COMPARISON OF GLUCAN AND MURAMYL DIPEPTIDE IN STIMULATION OF PARTICLE CLEARANCE AND HOST DEFENSE IN MICE

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The purpose was to determine if there is a positive correlation between the ability of 2 immunostimulants, Glucan and muramyl dipeptide (MDP), to enhance the rate of intravascular particle clearance in mice and to protect them against infections. Glucan treatment consisted of i.v. injections given 7, 4 and 1 days prior to the determination of carbon clearance rate. The MDP was given i.v. 1 day before rate determination. The dose that induced the greatest increase in carbon clearance rate was 40 mg/kg for Glucan and 8 mg/kg for MDP. These doses were used to pretreat mice that were inoculated i.v. with *Candida albicans* or *Staphylococcus aureus*. The survival time of MDP-treated mice, but not Glucan-treated mice, was significantly greater than untreated mice even though Glucan enhanced carbon clearance more than MDP.

ACKNOWLEDGMENTS

I am grateful for the guidance of John E. Lund, my mentor, and the financial support of The Upjohn Company. I thank Gary D. Gray, Karen Knight, Paul Skinner, W. Pat Brown, and Lori Harris for technical assistance. My guidance committee, Martha Thomas, Charles Whitehair, George Padgett, and Ronald Patterson, gave me valuable advice.

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INTRODUCTION

Agents that modulate host defense are of great importance because they may be useful in the prevention and/or treatment of infectious diseases or neoplasia. Glucan and muramyl dipeptide (MDP) are two compounds which have been reported to activate macrophages and to be effective in treating various experimental infections (1,2). They are considered to be good candidates for immunotherapy because they are non-antigenic and have low toxicity (1,3). Both Glucan and MDP increase the rate of intravascular carbon clearance in mice. The rate of carbon clearance is determined by administering carbon particles intravenously and measuring the relative carbon concentration in serial blood samples (4). The enhancement of carbon clearance may indicate therapeutic potential because carbon particles are primarily sequestered by macrophages in the liver and spleen and increased uptake is associated with activation of the macrophages.

The purpose of this research was to determine if there is a positive correlation between the ability of an agent to enhance the rate of carbon clearance and to protect the host against bacterial and mycotic infections. The dose-response relationship of Glucan and MDP treatment of mice to the rate of carbon clearance was determined. The protective activity of Glucan and MDP was determined by inoculating treated and untreated mice with *Staphylococcus aureus* and *Candida albicans*. The value of drug treatment was correlated with carbon

clearance stimulation. Other host defense factors were examined to further characterize the response to MDP and Glucan treatment. These included the serum levels of lysozyme and interferon and the gross and histologic appearance of the liver and spleen.

LITERATURE REVIEW

Muramyl dipeptide and Glucan are structurally related to the peptidoglycan cell wall of microorganisms. Like certain bacterial preparations, they have been reported to stimulate nonspecific resistance to infection in animals (2,5). Glucan has also been effective in the treatment of syngeneic tumors in animals (6) and metastatic tumors in man (7). Both have low toxicity and therefore are considered to be potential therapeutic agents (1,7). Although previous research has not evaluated these agents simultaneously, it has revealed many important similarities and differences.

Muramyl dipeptide is a water-soluble glycopeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine) that was synthesized to identify the active structure in Freund's complete adjuvant (8). Glucan is an insoluble polysaccharide (β-1,3 polyglucose) that was extracted from *Saccharomyces cerevisiae* and identified as the component of zymosan which produced hyperplasia and hyperfunction of the reticuloendothelial system (RES) (9). Treatment with MDP has produced hyperfunction of the RES without inducing hepatomegaly and splenomegaly (10). Hyperfunction of the RES refers to increased rate of clearance of foreign particles. Colloidal carbon has been used to measure the rate of clearance, which purportedly indicates the phagocytic state of the RES (11), although clearance rate may also reflect blood flow and platelet function (12). Glucan has been reported to increase the

rate of carbon clearance 10-fold (11). The greatest increase reported for MDP was 4-fold above that for untreated animals; however, doses in excess of 200 μg/mouse were not examined (13). The clearance rate remained elevated for 3 days following MDP treatment and 15 days following Glucan treatment.

Glucan treatment enhanced the primary and secondary antibody response to sheep erythrocytes in mice (14). This adjuvant activity was assumed to be mediated by macrophages because of the effects of Glucan on the RES.

The adjuvant activity of MDP has been more thoroughly studied (1). In a water-in-oil emulsion, MDP was as active as whole mycobacteria in the induction of delayed-type hypersensitivity and experimental autoimmune diseases. Primary and secondary antibody responses were increased when MDP was administered in saline both parenterally and orally. Many analogs of MDP have been synthesized and examined for adjuvant activity. Small structural changes can render the molecule inactive (8).

The adjuvant activity of MDP in various *in vitro* systems indicated that it acted directly on T and B lymphocytes (15). Although some workers have concluded that macrophages were not the target cell for the adjuvant activity of MDP (15), others have demonstrated otherwise (16).

Macrophages are important for natural resistance to infection. The enhanced nonspecific resistance to infections in animals with acquired cellular immunity is mediated by macrophages that have an increased microbicidal capacity (17). Treating macrophages *in vitro* with MDP increased phagocytosis and killing of bacteria as well as adherence to glass and cell spreading, phenomena associated with

increased killing capacity (18,19). *In vitro* stimulation of peritoneal macrophages with MDP increased colony stimulating activity, collagenase production, prostaglandin production, and intracellular cyclic AMP (1). Monocytes and macrophages secreted endogenous pyrogens and lymphocyte activating factor in cultures treated with MDP (1). Macrophages exposed to MDP *in vitro*, but not *in vivo*, inhibited the growth of mastocytoma cells (20) and the presence of MDP increased the cytolytic activity of macrophages from established cell lines and from murine sarcoma virus induced tumors (21).

The effects of Glucan on macrophages have been determined by examining cells from Glucan-treated animals. For example, the effect of Glucan on Kupffer's cells was examined histologically in rats treated with Glucan (22). Three days after 5 daily i.v. injections many Kupffer's cells had vacuoles containing Glucan. Kupffer's cells were increased in size and number and many granulomas were present in the hepatic sinusoids. Peritoneal macrophages from mice treated with Glucan intraperitoneally have also been examined (23,24). They exhibited increased size, spreading, adherence, chemotactic activity, acid phosphatase production, and antitumor cytotoxicity.

Lysozyme, an enzyme which lyses certain bacteria, is produced by macrophages. Glucan treatment has been reported to double serum lysozyme levels in mice (25). Although it has been reported that endotoxin stimulation of macrophages has a small effect on *in vitro* lysozyme production (26), the determination of serum lysozyme levels was suggested to be useful for monitoring *in vivo* macrophage stimulation and depression (25). Serum lysozyme values were interpreted as an expression of macrophage activity rather than macrophage number

because methyl palmitate, an RES depressant, inhibited the Glucan-induced increase in serum lysozyme (25).

In view of the ability of MDP and Clucan to stimulate intravascular particle clearance, immune responses, and macrophage activities, it is not surprising that these agents enhance nonspecific resistance to infection. Treatment with MDP increased host resistance to *Candida albicans* (27,28), *Klebsiella pneumoniae* (5), and *Trypanosoma cruzi* (29) [MDP has been used as an adjuvant in vaccines for *Plasmodium falciparum* (30) and influenza (31)]. The results of one study indicated that the best treatment regimen was 4 daily injections of MDP, 80 mg/kg, prior to an i.v. injection of a lethal dose of *C. albicans* (28). In another study MDP was most effective when given once or twice several hours before inoculation or when given concurrent with *C. albicans* (27). In the latter study, when mice were given an LD_{100} of *C. albicans*, 50% of the MDP-treated mice survived; when given an LD_{50} nearly 100% of the treated mice survived. Pretreating mice with MDP protected them against an i.v. challenge of 10^3 *K. pneumoniae* (5). Mice that were treated on day -1 with MDP received either 10, 100, or 1000 µg/mouse i.v. or 2000 µg/mouse orally. On the eighth day following inoculation, 33% of the mice were still alive in the group given 10 µg, 73% were alive in the group given 100 µg, 88% in the group given 1000 µg, and 63% in the group that was treated orally with 2000 µg of MDP. The percent of mice that survived did not change after day 8. Ten percent of the untreated control mice were alive on the eighth day.

Although treatment with MDP on the day prior to infection was effective in protecting mice against *Klebsiella* infection, it did not enhance resistance to *Trypanosoma cruzi* in a study where 7 different

MDP treatment regimens were employed (29). Pretreatment on day -2 was effective and continuous infusion via subcutaneous osmotic minipumps during days -2 through +4 was also effective.

Research on the fate of ^{14}C-MDP administered i.v. indicated that 75-95% is excreted in the urine unchanged within 2 hours after injection (32). This suggested that pretreatment induced changes in host defense systems that developed and endured after most of the MDP had been eliminated.

Glucan therapy enhanced nonspecific resistance to *C. albicans* (2), *Staphylococcus aureus* (33,34), *Francisella tularensis* (35), *Leishmania donovani* (36), and *Sporothrix schenckii* (37). Glucan pretreatment, but not post-treatment, reduced mortality in mice that were given 3 x 10^6 *C. albicans* i.v. In 12 days 10% of the pretreated mice died and 50% of the untreated control mice died. Pretreatment consisted of an i.v. injection of Glucan particles suspended in saline, 0.45 mg/mouse, on days -7, -4, and -1.

Experimental *S. aureus* infections in normal, leukemic, and cyclophosphamide-treated mice have been beneficially altered by Glucan pretreatment (33,34,38). In 3 experiments with normal mice, 100% of the untreated mice were dead by 6-14 days. In each experiment approximately 60% of the Glucan-treated mice were alive when the last untreated mouse died. Two i.v. pretreatment regimens were used: 0.45 mg/mouse on days -10, -7, -4, and -1; and 1.0 mg/mouse on days -7 and -4, with the challenge of 10^9 *S. aureus* i.v. on day 0. The beneficial effect of Glucan treatment was not absolute. Death loss continued in the treated groups until all mice were dead on day 26.

Glucan and MDP can stimulate immune responses, macrophage activities, and resistance to infection, but the composition and kinetics of the two drugs are different. These potentially therapeutic compounds cannot be compared using literature reports because studies used different strains of mice or infectious agents. Additional research is needed to compare these compounds and to determine the relationship of their protective activity to their ability to stimulate the rate of carbon clearance.

OBJECTIVES

The objectives of this research were:

1. To determine the optimum dose for enhancing carbon clearance in CF-1 mice with MDP and Glucan.

2. To determine the effect of the optimum dose on the survival of mice inoculated with *C. albicans* and *S. aureus*.

3. To determine the serum lysozyme value for mice treated with Glucan and MDP.

4. To determine the effect of drug treatments on weight and histological appearance of liver and spleen.

5. To determine if interferon is induced by Glucan treatment.

MATERIALS AND METHODS

I. Effect of Glucan and MDP Treatment on the Reticuloendothelial System

Materials

Mice: Female CF-1 mice were obtained from Charles River Breeding Laboratories (Portage, MI). They were 6-10 weeks old (15-24 g) and were fed Rodent Laboratory Chow 5001 (Ralston Purina Co., St. Louis, MO) and water *ad libitum*.

Glucan: Sterile Glucan, 10 mg/ml in 5% dextrose water, lot E0737 (Accurate Chemical and Scientific Corp., Hicksville, NY) was diluted to the desired concentration with Lactated Ringer's (Abbott Laboratories, Chicago, IL) and sonified 1 min with a Branson Sonifier set at power level 1 (Model S125 with standard tapered microtip).

Muramyl dipeptide: Lyophilized MDP (Groupment d'Interet Economique - Institut pour la Recherche et la Production d'Immunostimulants, Paris), lot 79001, was dissolved in Lactated Ringer's.

Colloidal carbon: Special ink, 140 mg/ml, lot C11/1431A (Gunther Wagner, Hannover, Germany), was diluted to a concentration of 16 mg/ml with Lactated Ringer's.

Protease solution: Thermolysin, 64 units/mg, lot 57C-0116 (Sigma Chemical Co., St. Louis, MO), was dissolved in water at a concentration of 63 µg/ml.

Lysozyme: The standard was egg white lysozyme, grade I, lot 57C-8025 (Sigma Chemical Co.) dissolved in phosphate buffer, pH 6.3, to make 5 standard solutions ranging from 0-20 µg/ml. *Micrococcus lysodeikticus*, lot 107C-0061 (Sigma Chemical Co.), was suspended at 100 µg/ml in phosphate buffer, pH 6.3 (K_2HPO_4, 0.036M; KH_2PO_4, 0.114M).

Spectrophotometry: For the carbon clearance test a Coleman Junior III spectrophotometer was used, wavelength 620 nm, with round, 10-mm cuvettes. For the serum lysozyme assay, a Beckman DU spectrophotometer was used, wavelength 540 nm, with square, 10-mm cuvettes.

Methods

All injections were given in the lateral tail vein at a constant volume of 0.01 ml/g body weight.

Carbon clearance test: The method of Halpern et al. (4) was modified by using protease solution instead of 0.1% Na_2CO_3 to lyse blood samples. Colloidal carbon, 160 mg/kg, was injected and at four 2.5 minute intervals blood was collected from the retro-orbital venous plexus using heparinized microhematocrit capillary tubes (Dade, Miami, FL). Occasionally fibrin clots formed while the sample was being transferred from the collection tube to a 50 µl capillary pipette (Drummond, Broomall, PA) prior to diluting. By using 2.5 ml of protease solution to dilute each sample, fibrin clots which may have formed were degraded. The absorbance of each of the 4 diluted samples was determined and plotted against time on semilog paper to

determine the half time of clearance, T/2. The clearance rate constant, K, was calculated by the equation: $K = \frac{\log 2}{T/2}$. Clearance was expressed as the mean rate constant, $\bar{K}$, of each group divided by the mean rate constant of the control group, $\bar{K}_c$.

Serum lysozyme: Serum or lysozyme standard, 0.1 ml, was mixed with 0.5 ml of the *Micrococcus* suspension at 25 C and the absorbance at 540 nm was recorded at 15 sec and 135 sec (39). The decrease in absorbance was calculated and converted to lysozyme concentration with a curve based on the standards.

Histology: Livers and spleens were weighed with an Arbor balance (model 306) and fixed in 10% neutral buffered formalin. Paraffin sections were prepared and stained with hematoxylin and eosin.

Statistics: Results were analyzed with the Student's t-test; $P<0.05$ was considered significant.

II. Experimental Infections

Materials

Pathogens: *Staphylococcus aureus* was obtained from Drs. N. R. Di Luzio and D. L. Williams (Tulane University School of Medicine, New Orleans, LA) and was assigned number UC6966 in The Upjohn Company Culture Collection (Kalamazoo, MI). *Candida albicans*, UC7165, The Upjohn Company Culture Collection, was originally isolated from a patient at Bronson Methodist Hospital (Kalamazoo, MI).

Phosphate Buffered Saline (PBS): The PBS, pH 7.2, contained NaCl, 0.137M; K_2HOP_4, 0.007M; and KH_2PO_4, 0.003M. It was sterilized by autoclaving for 20 minutes at 121 C.

Osmotic Minipumps: Alzet pumps (model 2001) were obtained from Alza Corp., Palo Alto, CA.

Methods

Minipump Implantation: The pumps were filled aseptically with 0.2 ml of MDP solution and were implanted subcutaneously in the scapular region in mice anesthetized with methoxyfluorane. Wounds were closed with metal clips.

Preparation of Inocula: *Candida albicans* was grown in Nutrient Broth (Difco, Detroit, MI) containing glucose (10 g/l) at 37 C for 18 hours on a shaker (200 rpm) (40). This yielded cultures containing 1-2 x 10^8 yeast/ml. Purity of the culture was verified by Gram's stain. Two milliliters of the culture was mixed with 10 ml PBS and centrifuged at 570 G for 15 minutes. The supernatant was discarded and the button was resuspended in PBS to a concentration of 6 x 10^6 Colony Forming Units/ml (CFU/ml). The CFU/ml was determined by diluting samples, in duplicate, to a concentration of approximately 7 x 10^3 yeast/ml with PBS and plating 0.1 ml on Sabouraud's Dextrose Agar (BBL, Cockeysville, MD) (40). After incubation at 37 C for 24 hours, the number of colonies on each plate was counted with an American Optical Colony Counter (Model 3327).

Staphylococcus aureus suspensions were prepared by the method of Di Luzio and Williams (33), except that the concentration of bacteria was determined from the absorbance of the suspension using a standard curve (Figure 13, Appendix). The curve was established with dilutions of 2 stock *S. aureus* suspensions in which the number of bacteria had been determined with a Petroff-Hausser counting chamber (Hausser

Scientific, Blue Bell, PA) using standard technique. The cultures were grown in Trypticase Soy Broth (BBL) for 18 hours at 37 C on a shaker at 200 rpm. This yielded cultures containing 1-3 x 10^9 bacteria/ml. Purity of the culture was verified by Gram's stain. The culture was washed 3 times with PBS in 15-ml tubes; centrifugation was 570 G for 20 minutes. After the first wash a sample was used to determine the bacterial concentration and after the third wash the cells were resuspended in PBS to a concentration of 5 x 10^9 bacteria/ml.

Mean survival time and statistics: Mean survival time was expressed as the Harmonic mean (Hm) which was calculated by the following equation: $Hm = n/(1/d_1 + 1/d_2 + \ldots + 1/d_n)$, where n is the number of mice in the group and d_1, d_2, ... d_n are days of death for individual mice (29). For surviving animals 1/d = 0. Mortality data were analyzed by the Mann-Whitney U test (two-tailed). Differences were considered to be significant if $P<0.05$.

III. Interferon

Materials

Cells: Mouse fibroblast L929, American Type Culture Collection (ATCC).

Virus: Vesicular Stomatitis Virus (VSV), ATCC, Indiana strain, was diluted to 250 plaque-forming units per ml with culture media.

Culture media: Minimum Essential Medium (MEM) with Earle's balanced salt solution (Microbiological Associates, Walkersville, MD) was used with the following added: 10% fetal bovine serum (Sterile

Systems, Logan, UT), 100 µ/ml Penicillin (Squibb, Princeton, NJ), 100 µg/ml Streptomycin Sulfate (Pfizer, New York, NY), and 5 µg/ml Amphotericin B (Squibb).

Interferon standard: A 100 µ/ml interferon standard was obtained from Dr. D. A. Stringfellow (The Upjohn Company) (41).

Agar: Equal volumes of a 1.5% solution of Noble agar (Difco) and a double-strength solution of culture media were mixed at 47 C.

Neutral red saline: NaCl, 0.16M; neutral red, 3 g/l.

Dulbecco's phosphate buffered saline (PBS), pH 7.2, was obtained from Grand Island Biological Co. (Grand Island, NY).

Method

Interferon was assayed by viral plaque reduction (41,42). Two milliliters of fibroblasts, 10^5 cells/ml, were put in each 35 mm well in culture plates (3506, Costar, Cambridge, MA) and grown to confluence. Six dilutions of each serum sample ranging from 1:10 to 1:5000 were prepared in MEM. The interferon standard was diluted 1:50, 1:100, and 1:500. Each dilution was incubated on 2 fibroblast monolayers, 1 ml/well, at 37 C with 5% CO_2. MEM was incubated on 6 monolayers as a control. After 24 hours of incubation the medium was removed and the cells were washed with 1 ml of PBS. Two-tenths milliliter of VSV were pipetted onto each monolayer and allowed to absorb for 1 hour. Agar was poured on the monolayers, 2 ml/well, and they were incubated 2 days and then stained with neutral red to facilitate plaque counting. Units/ml of interferon was defined as the reciprocal of the dilution which had half of the number of plaques that were present in the control wells.

RESULTS

I. Effect of Glucan and MDP Treatment on the Reticuloendothelial System

Carbon clearance was determined on day 0 in mice, 5-6 per group, that were injected with MDP on day -1 or with Glucan on days -7, -4, and -1. The doses were 0, 0.8, 2.5, 8.0, and 25 mg/kg for MDP and 0, 4, 13, 40, and 80 mg/kg for Glucan. Control mice (dose = 0) were given Lactated Ringer's. Clearance ($\overline{K}/\overline{K}_c$) increased as the MDP dose increased up to 8 mg/kg, where the maximum clearance was observed, 2.1 ± 0.7 (approximately twice the clearance of control mice and statistically significant). The clearance at the highest MDP dose was 1.5 ± 0.4 (Figure 1). The clearance in mice treated with Glucan increased to a maximum of 4.9 ± 1.8 at the 40 mg/kg dose and declined to 1.5 ± 0.4 at the 80 mg/kg dose. The increase in clearance that occurred with Glucan doses of 13 mg/kg and 40 mg/kg was significant, compared to the control (Figure 1). The half times are listed in Table 2 in the Appendix.

Serum lysozyme was determined on day 0 in mice, 5 per group, that were injected with MDP on day -1 or with Glucan on days -7, -4, and -1. The doses were 0, 8.0 and 25 mg/kg for MDP and 0, 40, and 80 mg/kg for Glucan. Serum lysozyme was not significantly different in groups treated with MDP and Glucan than it was in control groups. Each group had approximately 1.5 μg/ml (Table 3, Appendix). The change in absorbance for the 20 μg/ml lysozyme standard in the assay was 0.050.

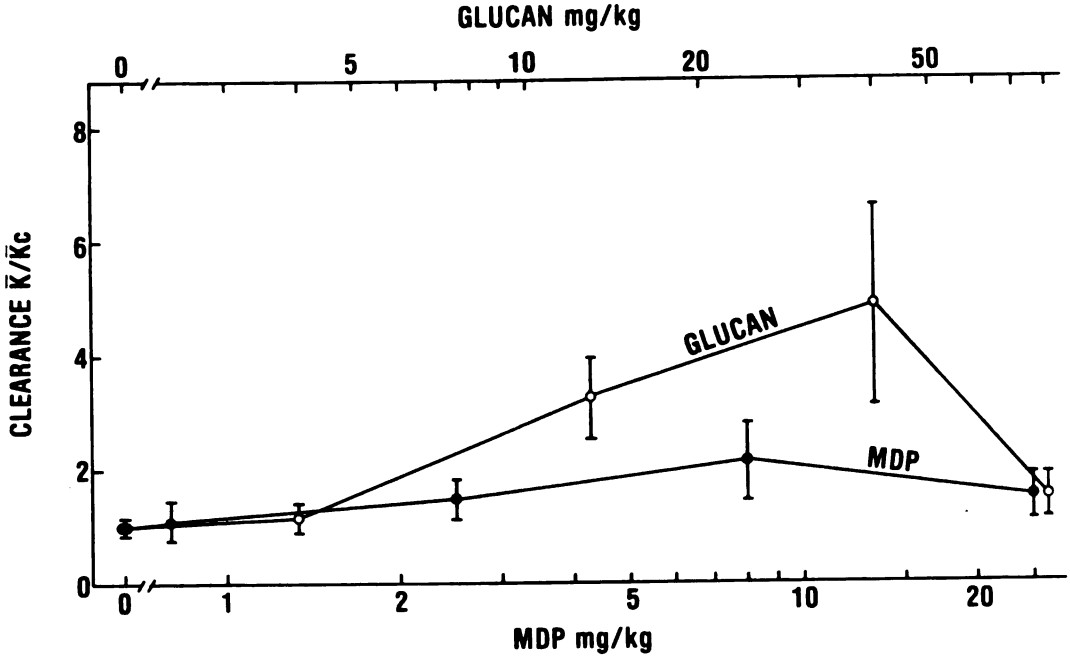

Figure 1. Effect of MDP and Glucan on carbon clearance in mice. Clearance was determined on day 0. The MDP was given on day -1. Glucan was given on days -7, -4, and -1. N = 3-6.

Four to six mice from each group in the carbon clearance experiment and the lysozyme experiment were killed immediately after blood samples were obtained and their livers and spleens were weighed. The comparison of organ weights of groups used for the carbon clearance test is termed "First experiment" and that of groups used for the lysozyme assay is termed "Second experiment." The MDP treatment resulted in significantly increased spleen weight in the first experiment only; at the dose of 8 mg/kg the spleen weight was 1.5 times that of the control. With Glucan treatment, spleen weights increased as the dose increased, and at the highest dose in the first and second experiments they were 3 and 2 times the control spleen weights, respectively (Figures 2 and 3). The increases observed in spleen weight with Glucan doses of 40 mg/kg and 80 mg/kg were statistically significant.

Liver weight increased to approximately 1.3 times the liver weight of control mice as the Glucan dose increased to 40 mg/kg, and it remained at that level at the highest Glucan dose, 80 mg/kg, in both experiments. The increase was statistically significant. The MDP treatment did not alter liver weight (Figures 4 and 5). Organ weights are listed in Tables 4 and 5 in the Appendix.

Livers and spleens from mice used in the lysozyme experiment were fixed in formalin and processed for histological examination. Many granulomas were observed in the livers of mice that were given Glucan doses of 40 mg/kg or 80 mg/kg on days -7, -4, and -1. Numerous granulomas were also observed in the spleens of mice treated at the highest Glucan dose. The spleens of mice that were given 40 mg/kg doses of Glucan had very few granulomas, but compared to controls they contained an increased number of vacuolated macrophages in the marginal

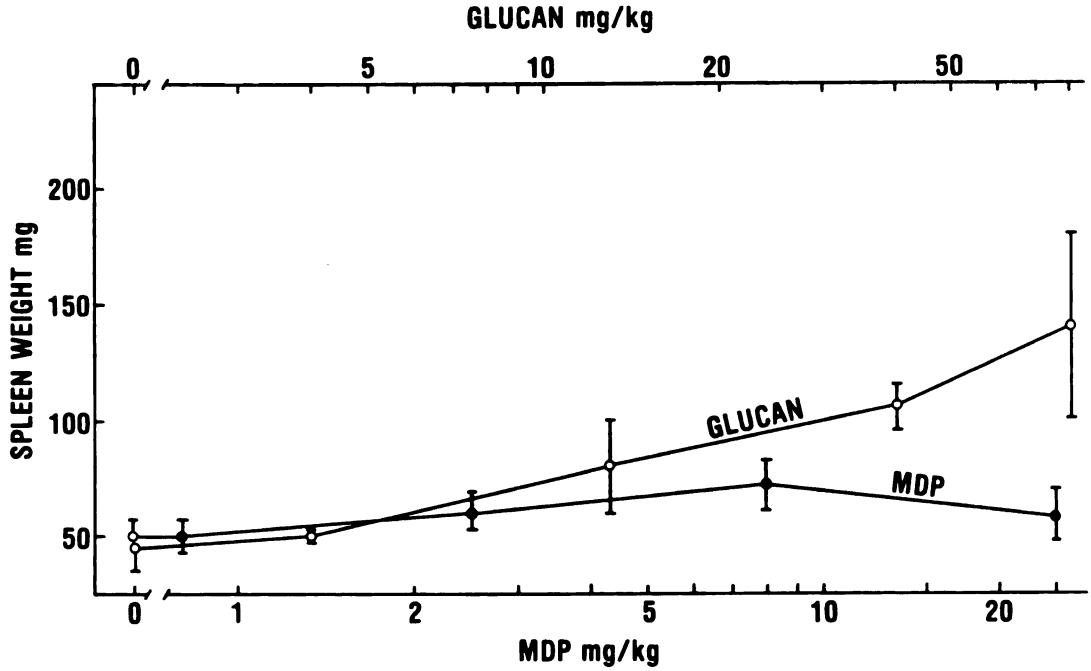

Figure 2. Effect of MDP and Glucan on spleen weight in mice. First experiment. Spleens were weighed on day 0. The MDP was given on day -1. Glucan was given on days -7, -4, and -1. N = 4-6.

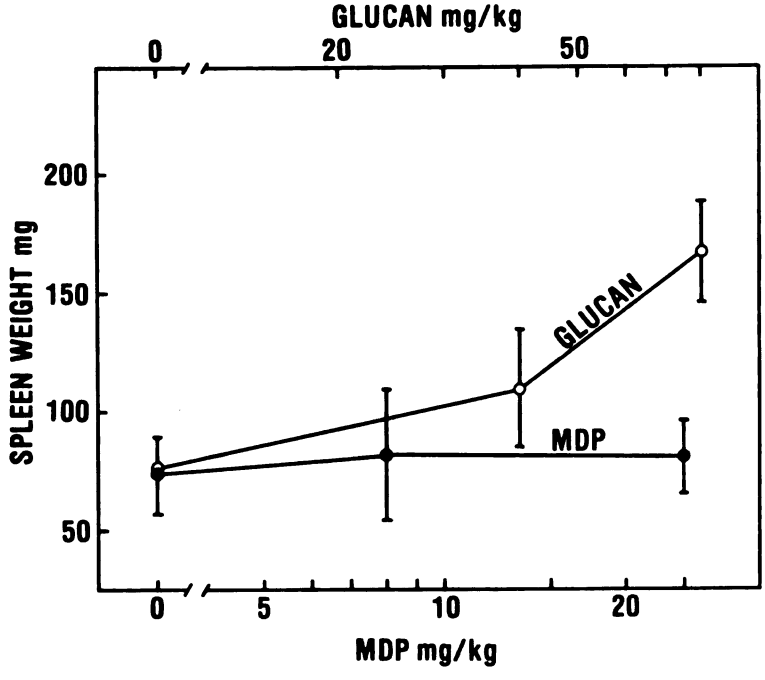

Figure 3. Effect of MDP and Glucan on spleen weight in mice. Second experiment. Spleens were weighed on day 0. The MDP was given on day -1. Glucan was given on days -7, -4, and -1. N = 5.

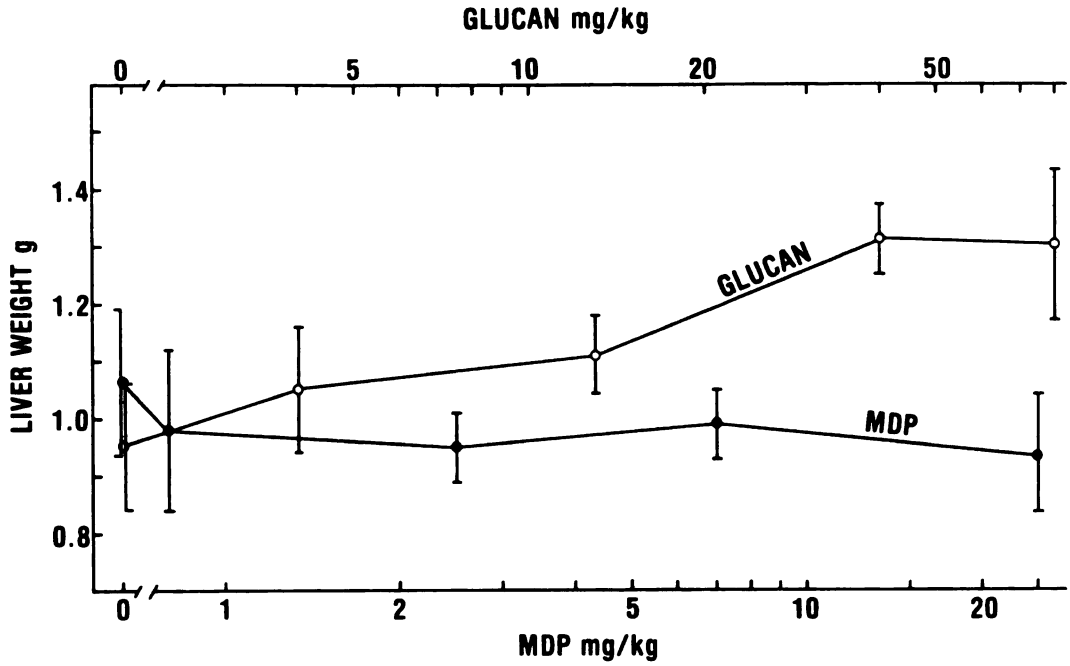

Figure 4. Effect of MDP and Glucan on liver weight in mice. First experiment. Livers were weighed on day 0. The MDP was given on day -1. Glucan was given on days -7, -4, and -1. N = 4-6.

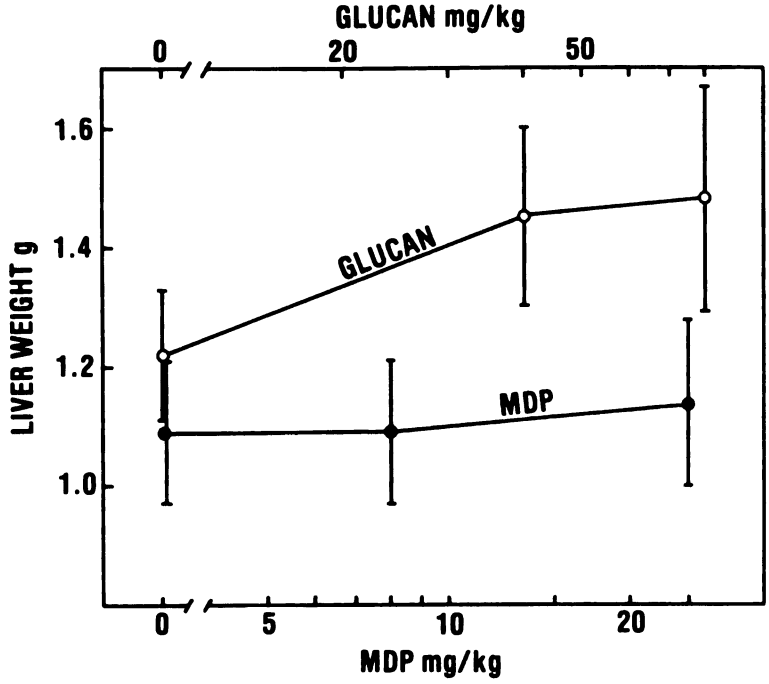

Figure 5. Effect of MDP and Glucan on liver weight in mice. Second experiment. Livers were weighed on day 0. The MDP was given on day -1. Glucan was given on days -7, -4, and -1. N = 5.

area of splenic nodules. The spleen and liver from mice treated with MDP appeared similar to the spleen and liver from control mice.

II. Experimental Infections

Two experiments were performed by pretreating mice and inoculating them on day 0 with 6×10^7 CFU/kg of *C. albicans*.

In the first experiment there were 5 groups of 10 mice. The control mice, group 1, were treated with Lactated Ringer's on day -2. The mice in group 2 were treated with Glucan, 40 mg/kg, on days -7, -4, and -1. Muramyl dipeptide, 8 mg/kg, was given to the mice in group 3 on day -2 and to the mice in group 4 on day -1. The mice in group 5 were implanted with Alzet minipumps on day -2. Each pump contained 200 µl of MDP at a concentration of 6.9 mg/ml. The delivery rate was calculated to be 8 mg/kg/day for 7 days. Only MDP, given on day -1 or by minimpumps, significantly increased survival (Figures 6 and 7). The Harmonic mean survival time (Hm) for groups 1, 2, 3, 4, and 5 was 4.5 days, 9.0 days, 11.5 days, 19.0 days, and 19.7 days, respectively.

To confirm the observation that MDP treatment on day -1 increased the survival of mice inoculated with *C. albicans* and the Glucan treatment did not, a second experiment was conducted with separate control groups for MDP and Glucan. Control mice, 10 per group, were given injections of Lactated Ringer's on day -1 (MDP control) or on days -7, -4, and -1 (Glucan control). Ten mice were given Glucan injections, 40 mg/kg, on days -7, -4, and -1, and 20 mice were given MDP injections, 8 mg/kg, on day -1. Only MDP significantly increased survival (Figures 8 and 9). The Hm was 4.7 days for the MDP control group and 16.6 days for the MDP treated group. The Hm for the Glucan control group was 14.9 days and for the Glucan treated group it was 6.4 days.

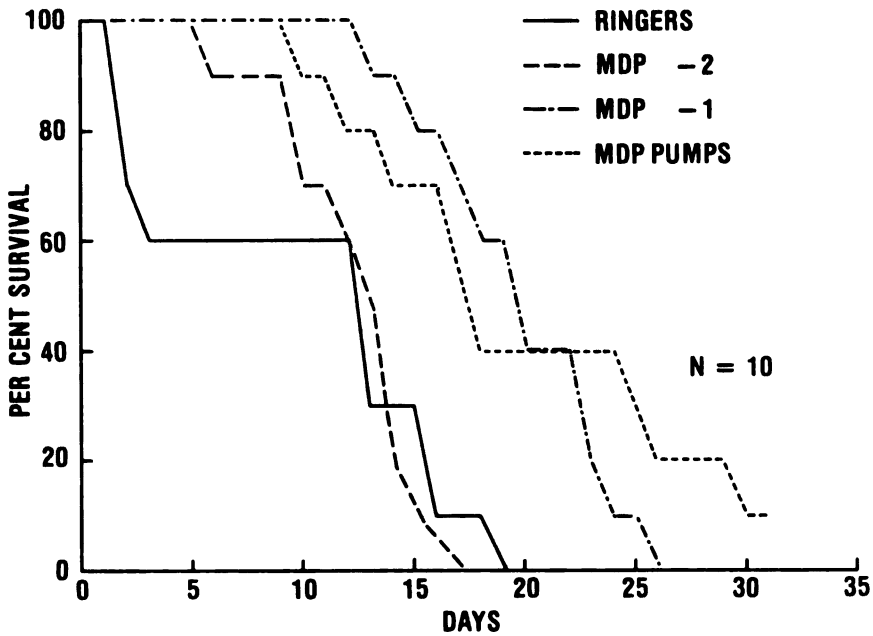

Figure 6. First *C. albicans* experiment. Effect of MDP, 8 mg/kg/day, given on day -1, day -2, or by minipump on days -2 through 4, on survival of mice inoculated with 6×10^7 CFU/kg on day 0.

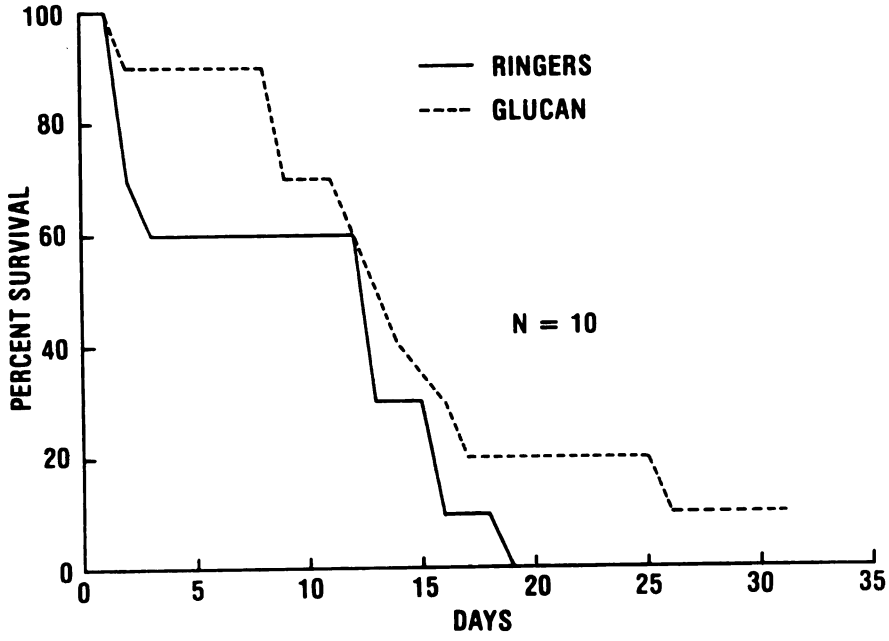

Figure 7. First *C. albicans* experiment. Effect of Glucan, 40 mg/kg, on days -7, -4, and -1, on survival of mice inoculated with 6×10^7 CFU/kg on day 0.

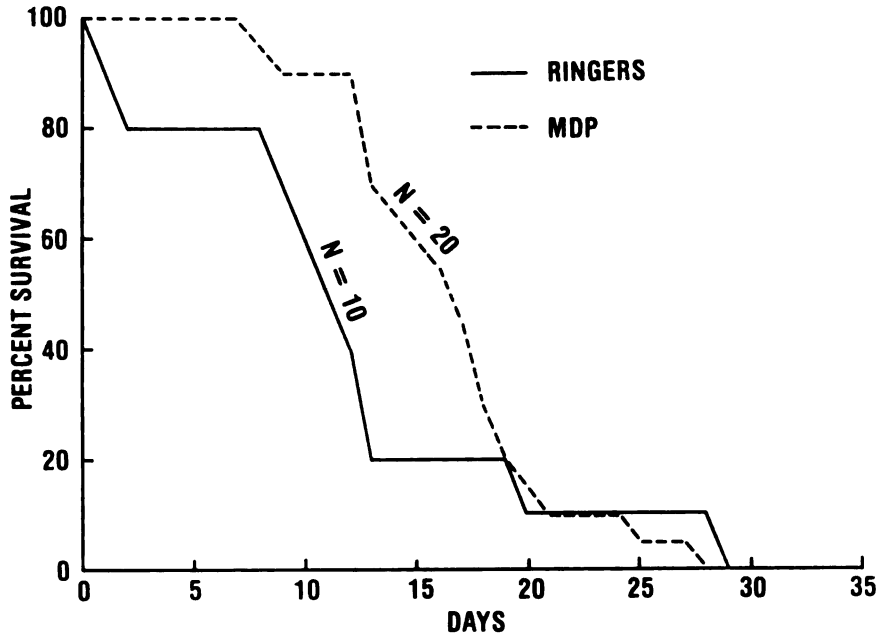

Figure 8. Second *C. albicans* experiment. Effect of MDP, 8 mg/kg, on day -1, on survival of mice inoculated with 6×10^7 CFU/kg on day 0.

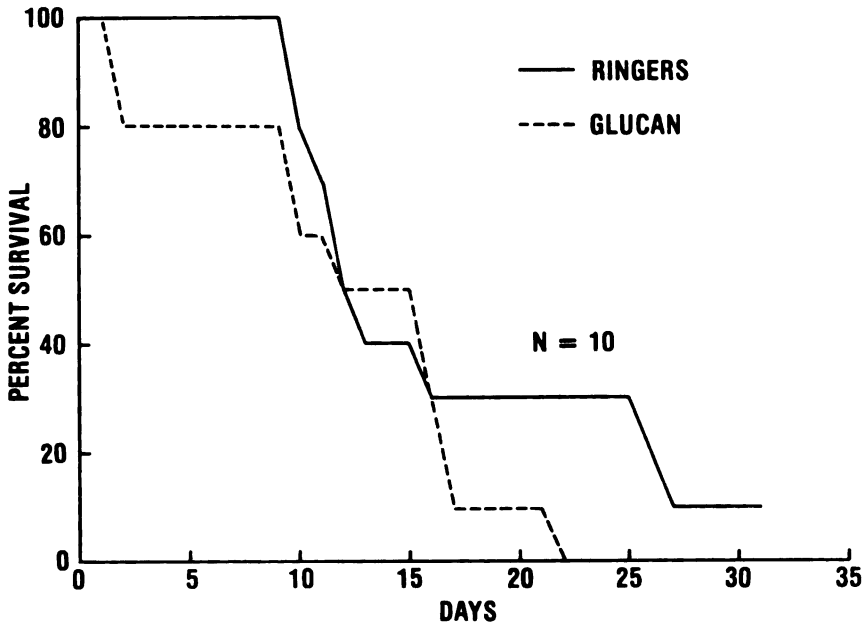

Figure 9. Second *C. albicans* experiment. Effect of Glucan, 40 mg/kg, on days -7, -4, and -1, on survival of mice inoculated with 6 x 10^7 CFU/kg on day 0.

Two experiments were performed by inoculating mice in groups of 20 with 5×10^{10} *S. aureus*/kg after pretreatment with Glucan or MDP.

In the first experiment mice in 2 groups were treated on days -7, -4, and -1 with either Lactated Ringer's or Glucan, 40 mg/kg. Mice in 2 other groups were treated on day -1 with Lactated Ringer's or MDP, 8 mg/kg. MDP treatment increased survival time significantly whereas Glucan treatment did not (Figures 10 and 11). The Hm was 4.4 days for the MDP control group, 23.3 days for the MDP treated group, 3.9 days for the Glucan control group, and 2.5 days for the Glucan treated group.

To determine if MDP given 2 days before inoculation would modify *S. aureus* infection, a second experiment was performed. Mice in 2 groups were treated on day -2 with Lactated Ringer's or MDP, 8 mg/kg. Mice in another group were treated on day -1 with MDP, 8 mg/kg. Both day -2 and day -1 MDP treatments significantly increased survival time (Figure 12). The Hm was 1.8 days for the control group, 4.1 days for the group treated on day -1, and 3.6 days for the group treated on day -2.

III. Interferon

Mice were bled at various times during Glucan treatment for serum interferon determination. Glucan, 40 mg/kg, was administered i.v. at time 0 and every 72 hours until mice were decapitated at the time indicated in Table 1. Lactated Ringer's was administered to control mice. Serum from 5 mice in each group was pooled and assayed for interferon. None of the sera from treated and control mice contained detectable levels ($\geq$10 units/ml) of interferon. However, the interferon level for the standard was less than 50 units/ml instead of the

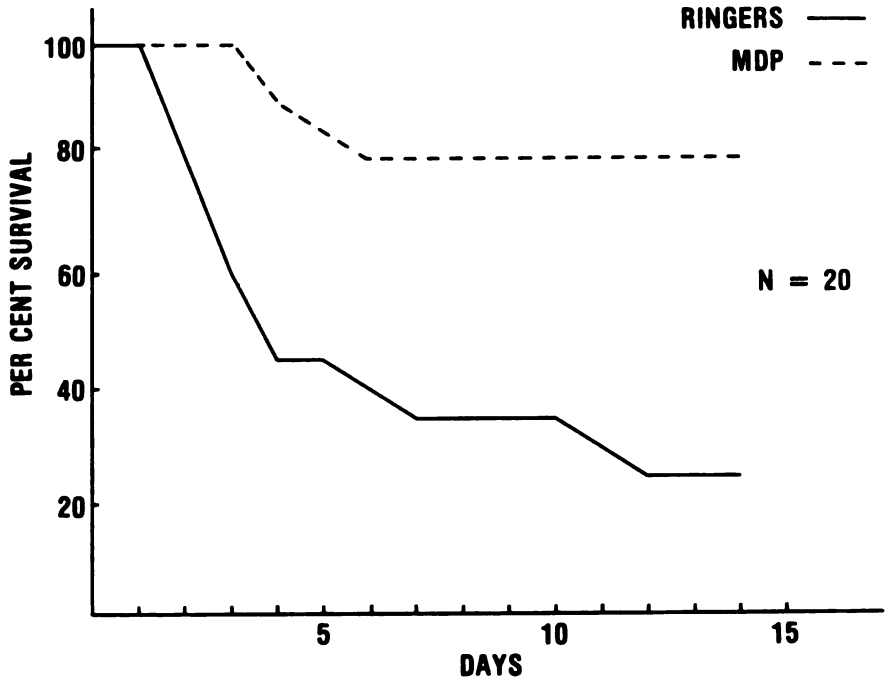

Figure 10. First *S. aureus* experiment. Effect of MDP, 8 mg/kg, on day -1, on survival of mice inoculated with 5 x 10^{10} bacteria/kg on day 0.

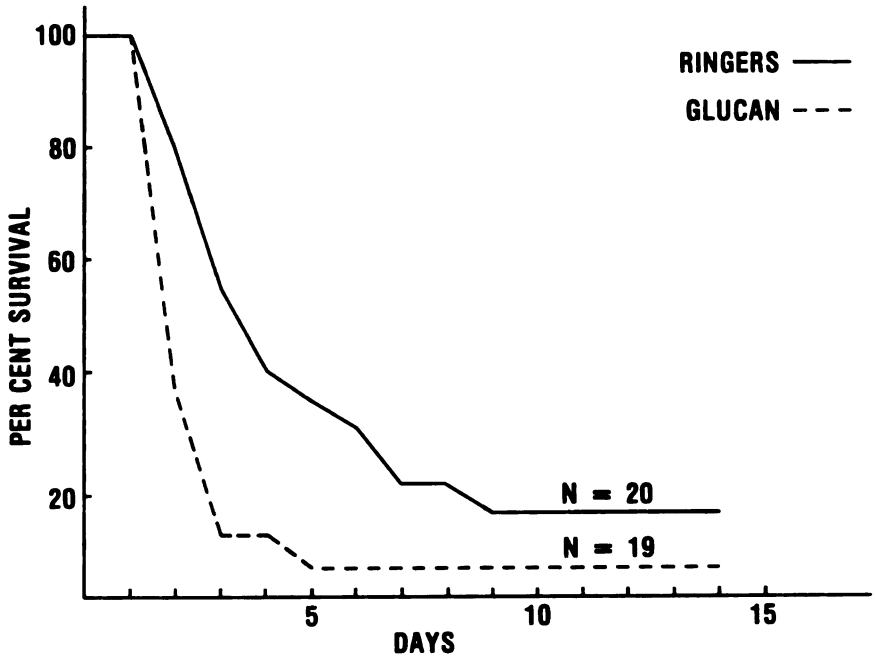

Figure 11. First *S. aureus* experiment. Effect of Glucan, 40 mg/kg, on days -7, -4, and -1, on the survival of mice inoculated with 5 x 10^{10} bacteria/kg on day 0.

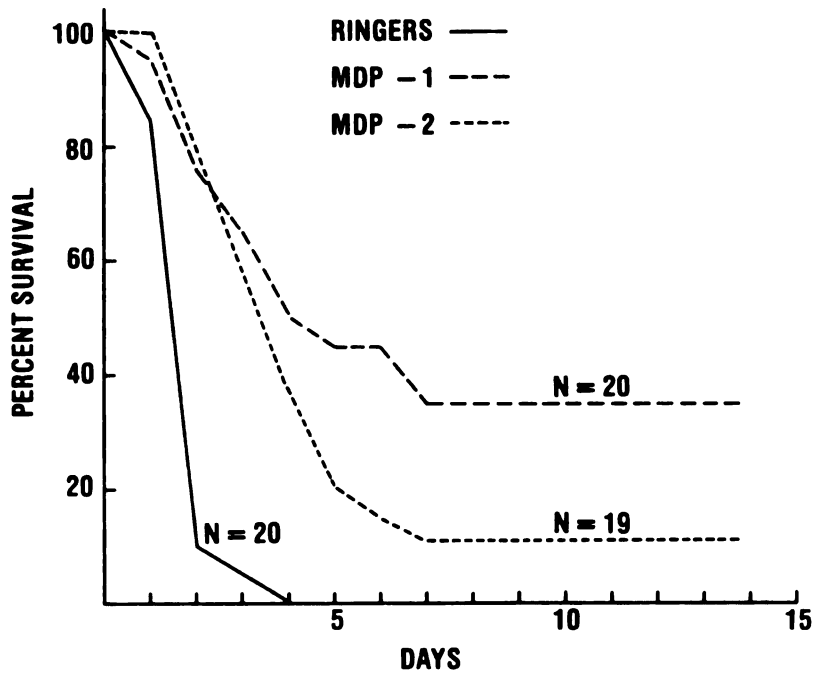

Figure 12. Second *S. aureus* experiment. Effect of MDP, 8 mg/kg, on day -1 or day -2, on survival of mice inoculated with 5×10^{10} bacteria/kg on day 0.

Table 1. Serum interferon sample times of mice treated with Glucan or Lactated Ringer's

Group[a]	Treatment[b]	Time of Sample (Hours)	Group	Treatment	Time of Sample (Hours)
1	Glucan	3	12	Glucan	84
2	Glucan	6	13	Ringer's	84
3	Ringer's	6	14	Glucan	96
4	Glucan	12	15	Glucan	120
5	Ringer's	12	16	Glucan	144
6	Glucan	24	17	Glucan	147
7	Glucan	48	18	Glucan	150
8	Glucan	72	19	Ringer's	150
9	Glucan	75	20	Glucan	156
10	Glucan	78	21	Ringer's	156
11	Ringer's	78	22	Glucan	168

[a]Five mice per group.

[b]Treatment began at time 0 and consisted of 1 i.v. injection every 72 hours until sample time.

expected value of 100 units/ml. To insure that the apparent lack of interferon in the serum samples was not the result of a decreased sensitivity of the assay, 4 serum samples that had reduced the plaque count by at least one-third were retested. Plaque counts again indicated that the pooled serum samples from Glucan-treated mice did not contain detectable levels of interferon. The value for the interferon standard in the second test was 100 units/ml, as expected. The plaque counts for these assays are listed in Table 6 in the Appendix.

DISCUSSION

The carbon clearance experiment indicated that the optimum Glucan dose for stimulating carbon clearance was 40 mg/kg. The large number of Glucan particles in the 80 mg/kg dose may have impaired phagocytosis. A 5-fold increase, observed at the 40 mg/kg dose, was also observed by Wooles and Di Luzio after 40 mg/kg doses were administered on days -3, -2, and -1 (11). They did not examine higher doses. We used different timing because our treatment was based on the report of Glucan treatment to protect mice against *C. albicans* by Williams et al. (2).

The optimum dose for stimulating carbon clearance with MDP was 8 mg/kg. The stimulation of carbon clearance with MDP at doses of 0 to 8 mg/kg has been reported before with results similar to ours (43).

The increased liver and spleen weight and granuloma formation observed in Glucan-treated mice confirmed reports that Glucan induced hypertrophy of the RES (11). It was reported that spleen and liver weight was not increased in mice treated with less than 200 µg of MDP (*ca.* 8 mg/kg) (10). In our research, liver weight was not significantly altered by MDP treatment. The effect of MDP treatment on spleen weight was not consistent. Spleen weight was significantly increased following 8 mg/kg of MDP in the First experiment. However, increased spleen weight was not observed in the Second experiment at MDP doses up to 25 mg/kg. Spleen weight may have been affected by carbon injections in the first experiment, but carbon was administered

to all groups and the weight of carbon injected was less than one-tenth of the spleen weight. Since increased spleen weight was not observed in the Second experiment, it is most likely that MDP treatment does not alter spleen weight.

In previous research, increased serum lysozyme was correlated with increased carbon clearance in rats and mice that were treated with Glucan (25). However, in this research with treatment methods that stimulated carbon clearance, neither MDP nor Glucan increased serum lysozyme levels. This discrepancy may have resulted from differences in the Glucan or the mouse strain used. Di Luzio used C57Bl/6J mice and 7.5 µg/ml was the normal serum lysozyme value for these mice (25). The serum lysozyme control value for the CF-1 mouse was 1.5 µg/ml, which may indicate decreased ability for lysozyme production and release in this strain. The source of commercially available Glucan is Di Luzio's laboratory, which uses a modification of the method of Hassid et al. to prepare Glucan (34,44). It was reported that the antitumor activity of Glucan from this source varies from batch to batch (45) and presumably other properties could also vary. The lot we used, E0737, may be unable to induce an increase in serum lysozyme. However, treatment with this lot did increase liver and spleen weight and carbon clearance. Di Luzio's hypothesis that serum lysozyme is a good index of macrophage stimulation is not supported by our research, which demonstrates that increased particle clearance can occur without an elevation in serum lysozyme.

Interferon levels also did not reflect macrophage stimulation. We postulated that Glucan treatment may induce interferon because Glucan is a polysaccharide that affects viral host defense. Polysaccharides have been reported to induce interferon which mediates

host defense against viruses and may also affect host defense against non-viral infections and neoplasia (46,47). However, interferon induction was not observed under the conditions of our research.

Glucan treatment did not significantly increase the survival time of infected mice even though our infection models were similar to those used by Williams et al. and Kokoshis et al. when they reported the protective effects of Glucan (2,38). The survival curves of control mice in our studies were similar to theirs and the dose, culture conditions and strain of *S. aureus* that we used were the same as theirs. The principal differences between our research and theirs were the lot and total amount of Glucan given and the strain of mouse used.

The potential differences in Glucan lots were discussed above. The total dose of Glucan in our studies was 120 mg/kg (3 x 40 mg/kg), the dose at which carbon clearance was maximally stimulated. A total Glucan dose of approximately 60 mg/kg (20 mg/kg on days -7, -4, and -1) was reported to increase the survival of mice inoculated with *C. albicans* (2). Protection against *S. aureus* was observed with total Glucan doses of approximately 80 mg/kg (34,38). The amount of Glucan that we administered may have been sufficient to "blockade" the RES and thereby eliminate some of the protective effects of Glucan. Although the carbon clearance data indicate that the dose would stimulate rather than "blockade" the RES, it is possible that Glucan treatment enhanced carbon clearance by mechanisms other than phagocytosis (12). The dose we used may have adversely affected bacterial uptake and killing.

It is possible that we did not observe enhanced host defense in Glucan-treated mice because we used a strain of mouse different than

those used in previous studies. The lysozyme results suggest that there is a strain difference in the response to Glucan treatment.

The MDP treatment which induced a 2-fold increase in carbon clearance always increased survival time significantly. This treatment, 8 mg/kg 24 hours before inoculation, was more effective against *S. aureus* than it was against *C. albicans* in terms of percent survival. Administering MDP 48 hours before inoculation instead of 24 hours reduced its effect against both infectious agents, especially *C. albicans*. This correlates with the reported observation that carbon clearance was elevated more one day after MDP treatment than it was at two days (43). The finding that resistance to *T. cruzi* was increased when MDP was given 48 hours prior to inoculation but not 24 hours (29) does not agree with our findings and suggests that defense mechanisms function differently in protozoal infections.

Administering MDP via minipumps (8 mg/kg/day, days -2 through 4) was no more effective than 8 mg/kg on day -1 against *C. albicans*. This agrees with a report that noted prolonged MDP treatment was not advantageous in *C. albicans* infection (27).

The mechanism of enhanced resistance to infection induced by MDP is not known. Presumably the compound interacts with macrophages directly to enhance phagocytosis and intracellular killing of microorganisms (19). Amplification of antibody responses by MDP may contribute to the destruction of any microorganisms that evade nonspecific defenses.

The rate of particle clearance is often used as an index of the phagocytic activity of reticuloendothelial cells (12,43). Agents that increase the rate of particle clearance are assumed to activate macrophages. They may increase the phagocytic rate by enhancing the

attachment phase or the ingestion phase of particle phagocytosis (48). However, macrophage number and other factors that contribute to particle clearance, such as particle aggregation, attachment of particles to platelets, and blood flow, may be affected by agents that increase the clearance rate (48).

Our research indicates that particle clearance is not useful for screening compounds to find agents that can stimulate nonspecific resistance to infections. Enhancement of the rate of carbon clearance did not corrleate with the status of host defense. Glucan treatment failed to increase resistance to infection even though it increased carbon clearance more than MDP treatment. The increased survival of MDP-treated mice demonstrates that the experimental infections used are susceptible to treatment and that host defense can be increased without inducing splenomegaly and hepatomegaly. Our results also indicate that serum lysozyme levels to not correlate with host defense status because MDP treatment did not increase serum lysozyme.

SUMMARY

The optimum dose of MDP and Glucan for enhancing the intravascular clearance of colloidal carbon injected in mice was determined. The effect of the treatments on resistance to infection was determined by inoculating mice with large doses, i.v., of *C. albicans* and *S. aureus*, after drug treatments, and observing their survival time. Serum lysozyme and interferon were assayed and liver and spleen were examined in treated mice.

For stimulating carbon clearance, the optimum Glucan dose was 40 mg/kg, i.v. (3 times), and the optimum MDP dose was 8 mg/kg, i.v. (1 time). Carbon clearance was increased 5-fold by the Glucan treatment and 2-fold by the MDP treatment. The survival time of MDP-treated mice, but not Glucan-treated mice, was significantly greater than control mice in 2 experiments with each infectious agent. The MDP was less effective when given 2 days prior to inoculation instead of 1 day. Serum lysozyme was not increased by either treatment. Glucan did not induce interferon but it did induce granulomatous hypertrophy of liver and spleen.

The status of host defense against bacteria and yeast did not correlate with the rate of carbon clearance or the concentration of serum lysozyme in mice. This indicates that the carbon clearance test and the measurement of serum lysozyme would not be useful for screening compounds for host defense-stimulating activity.

APPENDIX

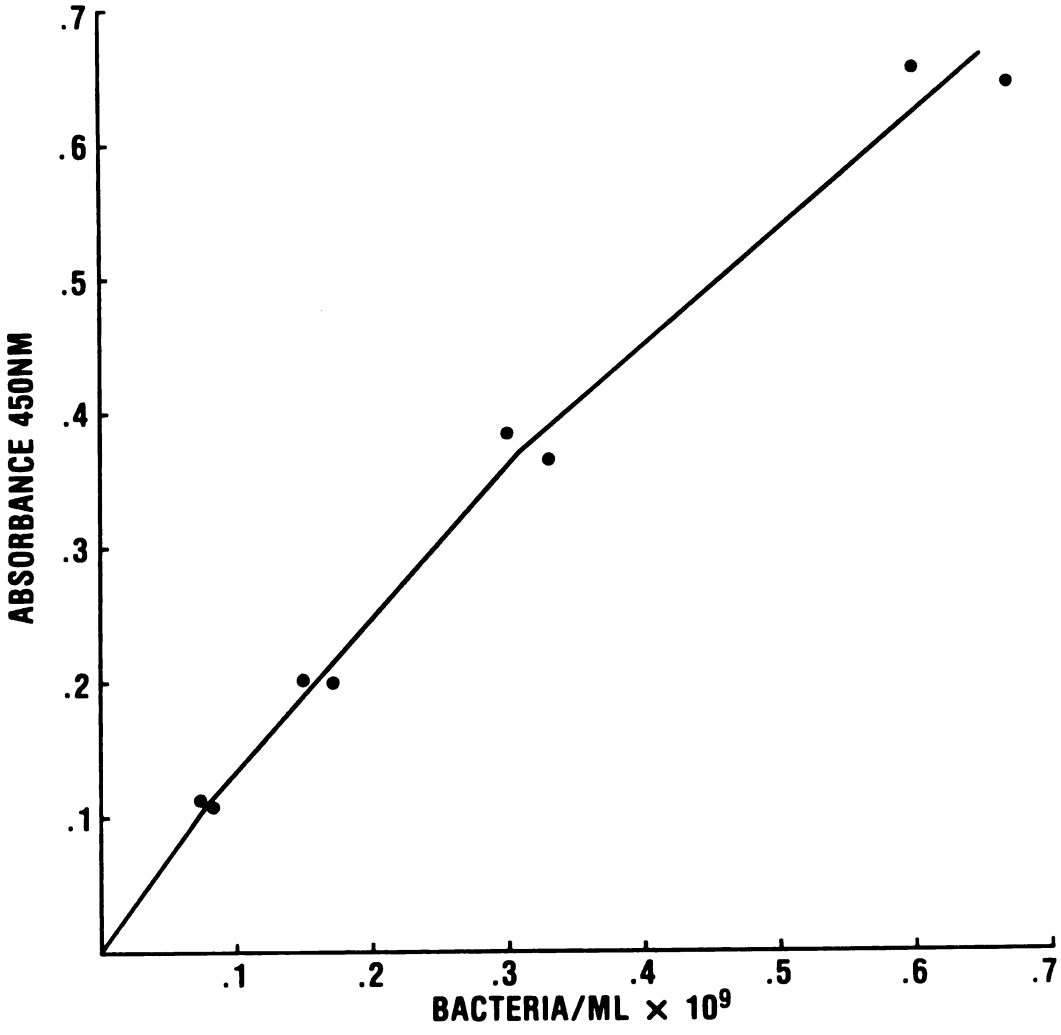

Figure 13. Standard curve for determining *S. aureus* concentration. Coleman Junior III spectrophotometer with 19 x 75 mm cuvette.

Table 2. Carbon clearance half times on day 0 of mice treated with Glucan and MDP

Treatment (mg/kg)	Half Times (Minutes)						Clearance $\bar{K}/\bar{K}_C$ Mean	S.D.
Glucan days -7,-4,-1								
0	8.2	9.9	6.8	6.4	8.0		1.00	0.17
4	7.3	8.5	7.9	6.2	5.1		1.14	0.24
13	1.9	2.8	2.6	*	*		3.24	0.69
40	3.7	1.1	2.1	1.4	1.5	1.3	4.87	1.75
80	6.0	6.2	5.4	3.7	*		1.51	0.39
MDP day -1								
0	8.6	8.0	10.1	6.2	6.8		1.00	0.19
0.8	5.9	8.8	8.7	7.8	8.2	4.5	1.12	0.34
2.5	4.0	6.5	6.2	6.9	4.5	4.6	1.48	0.34
8.0	2.3	5.6	4.2	4.0	3.3	4.2	2.12	0.68
25.0	7.6	4.3	6.3	4.1	4.4		1.52	0.41

*Blood samples were not obtained.

Table 3. Serum lysozyme on day 0 of mice treated with Glucan and MDP

Treatment (mg/kg)	Lysozyme (µg/ml)					Mean	S.D.
Glucan days -7,-4,-1							
0	5.0	1.6	1.2	1.6	0.0	1.9	1.9
40	0.0	1.6	1.2	1.2	1.2	1.0	0.6
80	0.8	2.3	1.2	0.0	0.8	1.0	0.8
MDP day -1							
0	2.7	0.4	0.4	1.6	1.2	1.3	1.0
8.0	1.6	0.8	1.9	1.6	0.0	1.2	0.8
25.0	1.6	0.0	2.7	1.6	0.4	1.3	1.1

Table 4. Spleen weights on day 0 of mice treated with Glucan and MDP

Treatment (mg/kg)	Spleen Weight (mg)						Mean	S.D.
First experiment								
Glucan								
days -7,-4,-1								
0	50	40	50	40			45	10
4	50	50	50	50			50	0
13	50	100	80	90			80	20
40	90	120	100	110			105	10
80	200	150	120	90	140		140	40
MDP								
day -1								
0	50	40	60	43	50	55	50	7
0.8	60	50	56	50	40	50	51	7
2.5	60	60	50	70	56	70	61	8
8.0	90	60	65	73	79	63	72	11
25.0	45	60	60	50	60	73	58	10
Second experiment								
Glucan								
days -7,-4,-1								
0	75	90	64	89	63		76	13
40	106	72	127	108	132		109	24
80	143	190	147	178	177		167	21
MDP								
day -1								
0	88	81	67	48	82		73	16
8.0	96	114	58	96	47		82	28
25.0	80	74	107	65	80		81	16

Table 5. Liver weights on day 0 of mice treated with Glucan and MDP

Treatment (mg/kg)	Liver Weight (g)						Mean	S.D.
First experiment								
Glucan								
days -7,-4,-1								
0	1.00	0.99	1.02	0.78			0.95	0.11
4	1.17	1.06	0.90	1.05			1.05	0.11
13	1.13	1.20	1.08	1.03			1.11	0.07
40	1.28	1.37	1.24	1.35			1.31	0.06
80	1.41	1.42	1.36	1.16	1.17		1.30	0.13
MDP								
day -1								
0	1.09	0.98	1.05	0.93	1.06	1.30	1.07	0.13
0.8	1.12	0.87	1.05	0.88	0.82	1.15	0.98	0.14
2.5	0.90	0.85	0.99	0.99	0.96	1.02	0.95	0.06
8.0	1.08	0.91	0.96	0.98	0.96	1.06	0.99	0.06
25.0	0.88	1.01	1.02	0.82	1.05	0.85	0.94	0.10
Second experiment								
Glucan								
days -7,-4,-1								
0	1.24	1.12	1.30	1.34	1.08		1.22	0.11
40	1.44	1.20	1.52	1.50	1.59		1.45	0.15
80	1.75	1.58	1.29	1.46	1.32		1.48	0.19
MDP								
day -1								
0	1.21	1.07	1.12	0.91	1.16		1.09	0.12
8.0	1.12	1.20	1.03	1.20	0.92		1.09	0.12
25.0	1.24	1.18	0.90	1.15	1.21		1.14	0.14

Table 6. Interferon assay plaque counts*

First Assay			Second Assay		
Group	Plaques/Well		Group	Plaques/Well	
	1:10 dilution			1:10 dilution	
1	20	32	6	24	34
2	27	36	7	33	34
3	32	33	8	25	32
4	26	31	9	30	31
5	31	37			
6	18	27		1:50 dilution	
7	20	29			
8	17	23	Standard	10	13
9	19	23			
10	29	31		1:100 dilution	
11	24	29			
12	19	32	Standard	19	20
13	26	23			
14	32	31	Virus		
15	28	36	Control	48,40,37	
16	27	24		35,37	
17	33	32			
18	23	24	Control		
19	24	27	Mean	39	
20	30	34			
21	27	28			
22	25	36			
	1:50 dilution				
Standard	19	20			
Virus					
Control	35,38,34, 24,30,31				
Control					
Mean	32				

*If a dilution had more than 1/2 the number of plaques in the control mean, higher dilutions were not counted.

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REFERENCES

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VITA

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I lived on a farm in Midland County, Michigan, from my birth, in 1951, until after I graduated from Coleman High School. I began studies in the School of Literature Science and Arts at the University of Michigan in 1969. Majoring in Medical Technology, I received clinical training at University Hospital. In 1973 I received my B.S. degree and passed the Medical Technologist Registry Examination of the American Society of Clinical Pathologists. During the next five years I was employed at various hospitals and clinical laboratories in Michigan. I was admitted to the Clinical Laboratory Science program at Michigan State University and received an Upjohn Fellowship from the Department of Pathology in 1978. My research was conducted at The Upjohn Company, Kalamazoo, Michigan, and I will receive my M.S. degree August 1980.