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Novel Immunomodulators With Pronounced In Vivo Effects Caused by Stimulation of Cytokine Release

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Abstract Beta-1,3-D-polyglucose derivatives protect mice against otherwise lethal bacterial infections. This protective effect has been considered to be mediated through mononuclear phagocytes. By using radioactive labelling, we localized the β-1,3-D-polyglucose derivatized microbeads (GDM) during the period following injection. The GDM was recovered mainly in the milky spots of the omentum. In animals treated with GDM, the total white cell number was significantly increased in peritoneal fluid of mice before and after challenge with *E. coli*. Bacterial counts in peritoneal fluid of GDM treated animals declined to zero after 24 h. In untreated animals there was a slight increase in bacterial counts until the animals died after about 12 h. Mouse peritoneal macrophages stimulated with GDM released significant amounts of IL-1 and PGE_2. There was no significant release of TNF. Levels of IL-1 and PGE_2 in peritoneal fluid increased significantly during the first 48 h after treatment with GDM. There was no increase of levels of TNF. After challenge with *E. coli*, the levels of IL-1, TNF, and PGE_2 were significantly lower compared with control animals. In untreated animals the levels of IL-1 and TNF remained elevated until the animals died after about 12 h. These studies demonstrate that the raised levels of arachidonic acid metabolites after pretreatment with GDM or AG seems to inhibit the otherwise lethal elevation of IL-1 and TNF in body fluids which is seen in untreated animals.

Key words: bactericidal activity, macrophages, neutrophils, β-1,3-D-polyglucose, microbeads, IL1, TNF, PGE_2

The immunoregulatory functions of mononuclear phagocytes are of crucial importance in both humoral [1,2] and cellular [3] immunity. These cells play a central role in inflammatory responses by secreting a variety of biologically active molecules, including a number of cytokines and metabolites of arachidonic acid [4,5]. We have shown that mononuclear phagocytes stimulated with β-1,3-D-polyglucose derivatives in vitro release increased amounts of these biologically active molecules [6]. We have recently reported that mononuclear phagocytes and granulocytes collected from animals treated with β-1,3-D-polyglucose derivatives have elevated bactericidal capacity [7]. It was demonstrated that this is related to the effects of an increased production of IL-1.

Beta-1,3-D-polyglucose derivatives protect mice against otherwise lethal bacterial infection [8,9]. The precise mechanism by which β-1,3-D-polyglucose derivatives exert this protective effect is not known. The development of sepsis is characterized by rapid changes in cellular, biochemical, and physiological parameters. Both IL-1, TNF and arachidonic acid metabolites are mediators in the pathogenesis of sepsis, but the pattern of interactions of these substances has not been delineated. In this study we have investigated the leucocyte counts, the bacterial counts, and the profile of IL-1, TNF, and PGE_2 in peritoneal fluid in untreated mice and mice pretreated with GDM before and after challenge with *E. coli*. It appears from our results that the β-1,3-D-polyglucose derivatives counteracts the lethal effects of sepsis at least partly by modulating the pattern of interactions of cytokines and arachidonic acid metabolites.

MATERIALS AND METHODS

Animals

Female CB6 F1 (Balb/C × C57BL/6) and C_3H/Hej mice were purchased from Gl. Bomholdtgård Ltd., Ry, Denmark. The mice were 5–6 weeks old when they arrived and weighed about 20 g. The mice were kept on standard laboratory chow and water, 3–5 animals per cage, for 1–3 weeks, including the experimental period. The

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animals were found to be free of common pathogens, including mouse hepatitis virus.

Treatment of Animals In Vivo

Animals were injected with 2×10^7 β-1,3-D-polyglucose derivatized microbeads (GDM) or 0.9% saline in a final volume of 1 ml into the peritoneal cavity 48 h before ip. injection of 10^8 live *E. coli*. In some experiments the GDM were preincubated with 10 μg/ml polymyxin B (Sigma Chemical Co., St Louis, MO) to exclude the possible contributions of contaminating LPS to the β-1,3-D-polyglucose induced inflammatory cell response. The C_3H/Hej strain, which does not respond to any contaminating LPS in the β-1,3-D-polyglucose derivatives, was used in some control experiments. Four mice in each group were sacrificed at the indicated timepoints. The experiments were repeated twice.

Preparation of Polyglucose Derivatives

Amination of β-1,3-D-polyglucose was performed as previously described [10]. Aminated monodisperse beads with a diameter of 4 μm prepared by copolymerization of 2,3-epoxypropyl methacrylate and ethylene glycol dimethacrylate [11] were obtained from Professors J. Ugelstad and A. Berge, University of Trondheim, Norway. Beta-1,3-D-polyglucose derivatized beads, 4 μm in diameter, were obtained as described previously [10,12]. The amount of covalently attached carbohydrate was determined by the phenol-sulphuric acid method [13] to be 7.5 μg/10^8 beads; 10^8 GDM had <0.1 ng LPS determined by thromboplastin activation [14]. Tritiated polyglucose derivatized microbeads were produced as follows: Hydrolyzed polyglucose was subjected to minimal oxydation with bromine [15] and, subsequently, was reduced again by sequential addition of radioactive sodium borotritide (New England Nuclear, Dreieich, Germany) and sodium borohydride (Merck, Darmstadt, Germany). The radioactive polyglucose was then attached to the beads by the same procedure, as previously described [10,12]. The specific activity was 2×10^5 cpm/10^8 beads, as determined with a liquid scintillation spectrometer (Packard Instruments, Zurich, Switzerland).

The content of radioactivity in the peritoneal organs was determined at given timepoints after perfusion of the organs with saline (to rid them of the blood bacteria component) and solubilization of the tissue in 1 N sodium hydroxide. The results are recorded as the percentage of injected radioactivity.

E. coli

A clinical isolate of serum resistant *E. coli* (serotype 018 ac: K 16: H 7) was passed twice by peritoneal infection in mice. The cultures were then expanded by incubation in lactose broth (LB, Difco Laboratories, Detroit, MI) [16] at 37°C for 1–2 h and stored frozen at −70°C for 6–12 months. Prior to injection into animals, the bacteria were rapidly thawed, washed twice in PBS (Dulbecco, Gibco, Biocult, Glasgow, UK), and kept on ice until use—for a maximum of 1–2 h. The number of bacteria was determined by microscopy immediately before injection into animals and by plating on agar.

Determination of *E. coli* in Peritoneal Fluid

Samples of peritoneal fluid were taken at the indicated timepoints, diluted 10-fold three times and plated in triplicate onto LB agar. The plates were incubated at 37°C for 24 h and the colonies were counted.

Determination of Total Leucocyte Levels and Differential Counts in Peritoneal Fluid

Peritoneal fluid was obtained by lavage with sterile saline. Four milliliters of saline were injected into the peritoneal cavity and the abdomen of the animal was gently massaged. The total cell number was counted by microscopy. Aliquots of 100 μl of lavage fluid were spotted onto glass microscope slides. Slides were stained with May Grunwald stain for 5 min. All slides were examined under oil immersion (100×) and were subjected to two separate microscopic screenings of 100 cells each. The number of each cell type per 100 cells was expressed as a percentage.

Isolation and Fixation of Milky Spots

The mice were sacrificed 48 h after intraperitoneal injection of GDM. The lesser omentum with milky spots was dissected free from the stomach and liver and subsequently spread out in a petri dish containing 2.5% glutaraldehyde (Merck, Darmstadt, Germany) with 0.1 M Na-cacodylate buffer and 0.1 M sucrose; *pH 7.4; 320* mOsm. at 4°C for 24 h. The specimens were dehydrated in a graded series of ethanol solutions and embedded in paraffin. For light micros-

copy, paraffin sections were stained with Hematoxylin/Eosin.

Chemicals

The following chemicals were used: RPMI 1640 (Gibco, Biocult, Glasgow, UK), synthetic serum replacement 2 (SSR 2) (Medi-Cult^R SSR, GEA-BioTech, Hvidovre, Denmark), lymphoprep (Nycomed AS, Oslo, Norway), instagel II (Packard Instruments, International, Zürich, Switzerland), protein-A Sepharose (Pharmacia, Uppsala, Sweden), MTT tetrazolium (M-2128 Sigma Chemical Co., St. Louis, MO), polyclonal rabbit anti-human interleukin-1 antibody (Genzyme Co., Boston, MA). The antibody did not demonstrate detectable reactivity with IL-2, IL-3, colony stimulating factor, or tumor necrosis factor. Ultrapure human IL-1 (Genzyme) had a specific activity of 8×10^6 U/μg protein and was added to the cultures at a final concentration of 0.1 ng/ml. It was free of endotoxin, IL-2, IL-6, and interferon gamma. Recombinant murine TNF alpha (Genzyme) was added to the cultures at a final concentration of 0.1 ng/ml. The specific activity for rTNF was 3×10^9 U/mg, LD_{50} of rTNF was 2×10^{-3} ng/ml. Polyclonal rabbit anti-murine TNF alpha (Genzyme) was used; interferon gamma (Amersham, Buckinghamshire, England) was added to the cultures at a final concentration of 10 ng/ml; radioimmunoassay kit was used for determination of PGE_2 in cellular supernatants (Amersham). Prostaglandin E_2 was added to the cultures at a final concentration of 10 ng/ml.

Preparation of Mouse Peritoneal Macrophage Cultures

Macrophages were collected by peritoneal lavage. The cells were washed twice in RPMI 1640 with Hepes buffer (20 mM) of pH 7.4 by centrifugation at 4°C at 200*g* for 10 min. After the washing, the cells were resuspended in RPMI 1640 with 0.1% SSR 2, seeded in 96-well, flat-bottomed microtitration plates at a concentration of 10^5 cells/well and cultivated as described above.

Determination of IL-1 in Macrophage Supernatants and Peritoneal Fluid

IL-1 activity was determined by evaluating the cytotoxicity of the IL-2 dependent HT-2 cell line [17] incubated with cell supernatant from the NOB-1 cell line [18], which produces IL-2 in response to IL-1 in test samples. NOB-1 cells were washed three times in RPMI 1640 (Gibco, Biocult, Glasgow, UK) and adjusted to 2×10^6 cells/ml in RPMI 1640 containing 5% FCS. The cells were distributed into 96-well microtitration plates at 2×10^5 cells/well. Cell supernatants and peritoneal fluid were added in three 10-fold dilutions to appropriate wells, and the volume made up to 200 μl with RPMI 1640 containing 5% FCS. In each experiment, a corresponding standard curve was made by adding ultrapure human IL-1 (Genzyme Co., Boston, MA) to control wells at varying concentrations. Ultrapure human IL-1 had a specific activity of 8×10^6 U/μg protein. It was free of endotoxin, IL-2, IL-6, and interferon gamma. The culture supernatants were harvested after 24 h incubation at 37°C and 5% CO_2. An 8-channel pipette was used to transfer 100 μl supernatant into a replicate microtitration plate. The contents of the plates were frozen above liquid nitrogen to kill any NOB-1 cells carried over in the supernatant. Forty microliters of each harvested supernatant were then added to microtitration wells containing 1.5×10^4 HT-2 cells in 60 μl of RPMI 1640 plus 5% FCS. The HT-2 cells were prepared by washing three times in RPMI 1640 to remove any IL-2 present in their maintenance medium. After 20 h of incubation at 37°C in 5% CO_2 in air, 10 μl of MTT tetrazolium (M-2128 Sigma Chemical Co., St. Louis, MO) at a concentration of 5 mg/ml in phosphate-buffered saline was added and further incubated for 4 h at 37°C. One hundred microliters of isopropanol with 0.04 N HCL was added to all wells and resuspended three times to disintegrate the cells. The plates were read on a Titertek Multiscan microplate reader at a wavelength of 540 nm. Survival of HT-2 cells was abolished by adding polyclonal rabbit anti-human interleukin-1 antibody (Genzyme) to test samples and IL-1 preparations in a final dilution of 1:200 one hour before the addition of NOB-1 cells. The antibody did not demonstrate detectable reactivity with IL-2, IL-3, colony stimulating factor, or tumor necrosis factor.

Determination of TNF in Macrophage Supernatants and Peritoneal Fluid

TNF activity in macrophage supernatants and peritoneal fluid was determined by use of the MTT tetrazolium cytotoxicity assay [19] with the TNF sensitive WEHI 164 clone 13 mouse sarcoma cells [20]. In brief, target cells were

seeded in 96-well, flat-bottomed microtitration plates at a concentration of 2×10^4 cells/well in 100 μl RPMI-SSR2 (synthetic serum replacement 2) (Medi-Cult[R] SSR, GEA-BioTech, Hvidovre, Denmark). Samples of macrophage supernatants or peritoneal fluid were added to the target cells in quadriplicates in a final concentration of 5%. The cytotoxicity was abolished by adding polyclonal rabbit anti-murine TNF alpha (Genzyme) to a number of cultures in final dilutions of 1:500. Obliteration of the cytotoxic effect was taken as a positive test for the presence of TNF. To obtain chemical amounts of TNF in each experiment, a percentage of dead cells were fitted to a standard curve made by adding rTNF (Genzyme) to control wells at varying concentrations. The specific activity for rTNF was 3×10^9 U/mg, LD_{50} of rTNF was 2×10^{-3} ng/ml. Incubation with RPMI-SSR2 was used as control. Recombinant TNF at a concentration of 10^{-4} ng/ml had a detectable cytotoxic effect on WEHI clone 13 cells. After 20 h of incubation at 37°C in 5% vol/vol CO_2 in air, 10 μl MTT at a concentration of 5 mg/ml in phosphate-buffered saline was added and further incubated for 4 h at 37°C. One hundred microliters isopropanol with 0.04 N HCL was added to all wells. Dark blue formazan crystals appeared, derived from MTT tetrazolium by dehydrogenase activity in living cells. The plates were read on a Titertek Multiscan microplate reader by using a test at a wavelength of 540 nm. The percentage of dead target cells was determined as:

$$\frac{\text{OD in wells with test sample/rTNF}}{\text{OD in control}} \times 100.$$

Determination of PGE_2 in Macrophage Supernatants and Peritoneal Fluid

The levels of PGE_2 in macrophage supernatants and peritoneal fluid was determined by using a radioimmunoassay kit (Amersham International, UK) [21] based on the conversion of 13,14-dihydro-15-keto-prostaglandin E_2 in test samples to 11-deoxy-13,14-dihydro-15-keto-11β,16E-cyclo-prostaglandin E_2 (bicyclic PGE_2) by sodium carbonate. All of the rapidly formed metabolites of 13,14-dihydro-15-keto-prostaglandin E_2 were converted to bicyclic PGE_2 (as determined by radioimmunoassay). Briefly, the assay utilized the competition between unlabelled 11-deoxy-13,14-dihydro-15-keto-11β,16E-cyclo-prostaglandin E_2 (bicyclic PGE_2) and a fixed quantity of the tritium-labelled compound for binding to a protein which had a high specificity and affinity for the bicyclic PGE_2 metabolite. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody was inversely proportional to the concentration of added non-radioactive ligand. Measurement of the protein-bound radioactivity enabled the amount of unlabelled bicyclic PGE_2 in the sample to be calculated. Separation of the protein bound bicyclic PGE_2 from the unbound ligand was achieved by adsorption of the free bicyclic PGE_2 onto dextran-coated charcoal, followed by centrifugation. Measurement of the radioactivity in the supernatant liquid quantitated the amount of radioactive ligand bound by the antibody. The concentration of unlabelled bicyclic PGE_2 in the sample was then determined from a linear standard curve.

Statistical Analysis

The analysis of differences between the groups was performed by means of a two-tailed t-test with N-2 degrees of freedom. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Protective Effects of GDM in *E. coli* Infection—Dose/Response Relationship

Pilot experiments established that the injection to the peritoneum of more than 5×10^7 *E. coli* invariably led to severe sickness and death of the animals within 12 h. However, eight animals treated with 10^8 β-1,3-D-polyglucose derivatized microbeads (GDM) 48 h before inoculation with *E. coli* showed no signs of disease and none of these animals were dead 1 week later. The experiments were repeated twice with the same result. Figure 1 shows that 1.5 μg β-1,3-D-polyglucose, corresponding to 2×10^7 beads/mouse, was the minimum quantity to achieve full protection. At doses lower than this there was a positive correlation between the dose of GDM and the survival rate.

Distribution of Intraperitoneally Injected GDM

Forty-eight hours after intraperitoneal injection of radioactive polyglucose derivatized microbeads, 75% of the injected radioactivity was recovered in the milky spots of the omentum (Fig. 2). About 10% remained in peritoneal cells and only small amounts were found in liver, spleen, kidneys, and lungs (data not shown).

Light Microscopy of Milky Spots

The connective tissue of the omentum was composed of a loose cellular reticulum sustained

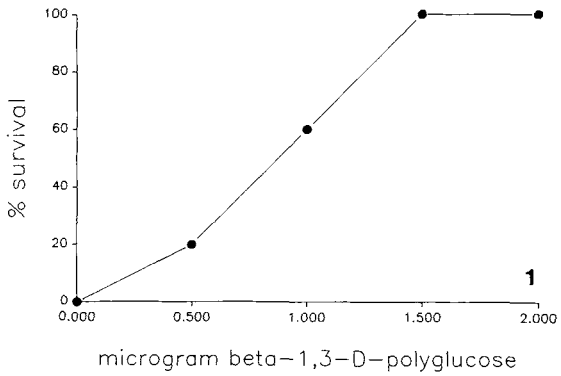


Fig. 1. Survival of mice treated 48 h earlier with various quantities of β-1,3-D-polyglucose derivatized beads and infected with 10^8 virulent *E. coli* injected into the peritoneum.

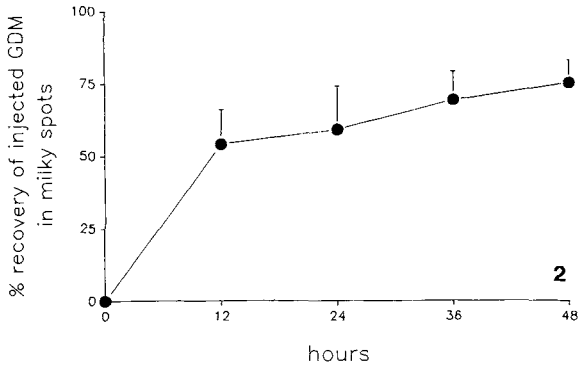


Fig. 2. Percent recovery in milky spots of the omentum of intraperitoneally injected GDM. The results are expressed as mean ± SD.

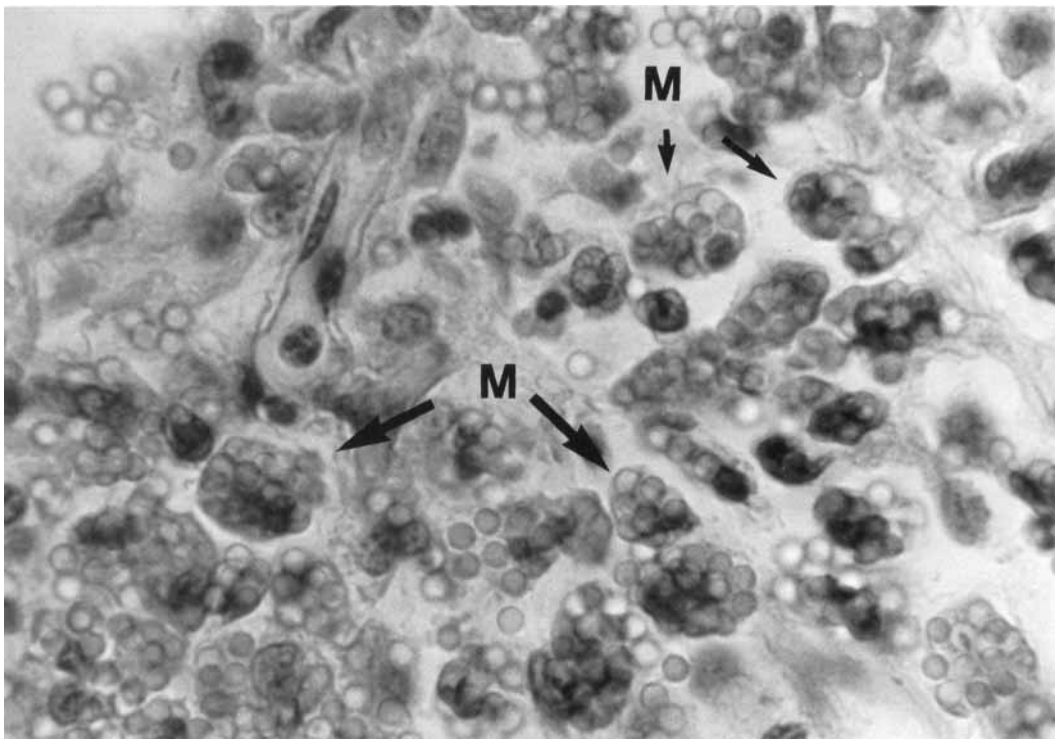


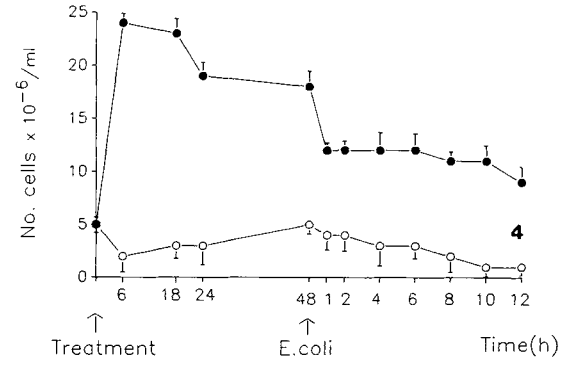
Fig. 3. Micrograph of a milky spot of mouse omentum 48 h after intraperitoneal administration of GDM showing macrophages with intracellular beads (M) (×280).

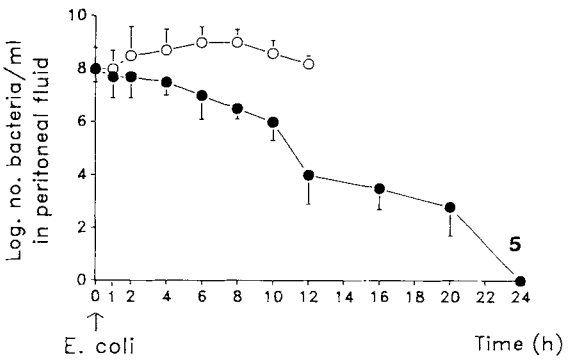
by fine collagenous fibers lined by a continuous layer of mesothelial cells. Sparse fat cells were separated by denser connective tissue. Aggregates of densely staining mononuclear cells—macrophages and lymphocytes (the milky spots)—were scattered throughout the omentum. The milky spots were vascularized and were only incompletely covered with mesothelial cells.

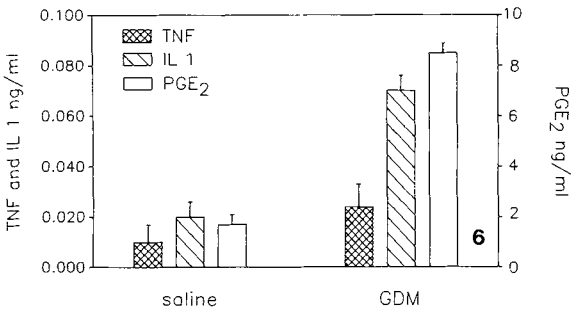
Microscopic examination of milky spots 48 h after intraperitoneal administration of GDM showed dense aggregates of macrophages with intracellular beads (Fig. 3).

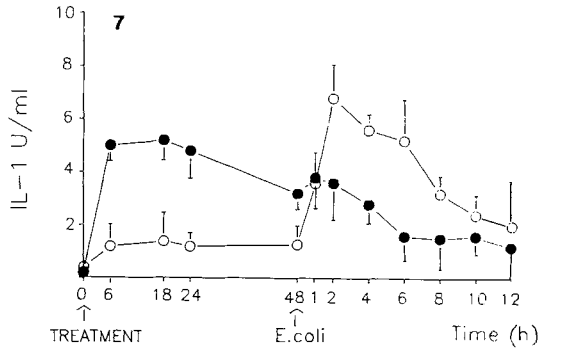
Effect of GDM on Peritoneal Leucocyte Levels Prior to and Following *E. coli* Challenge

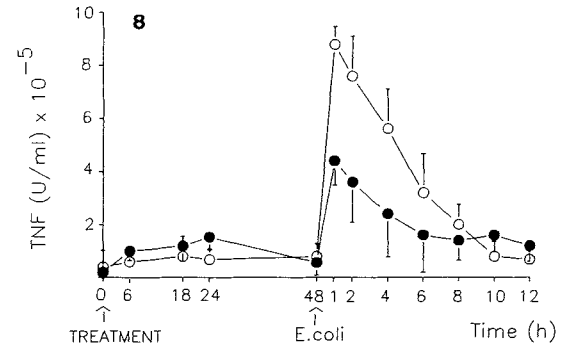
Intraperitoneal administration of β-1,3-D-polyglucose derivatized microbeads resulted in a 4–5 times increase in number of total peritoneal leucocytes after 6–18 hours (Fig. 4). The elevated cell number was mainly due to increased levels of granulocytes, although a significantly











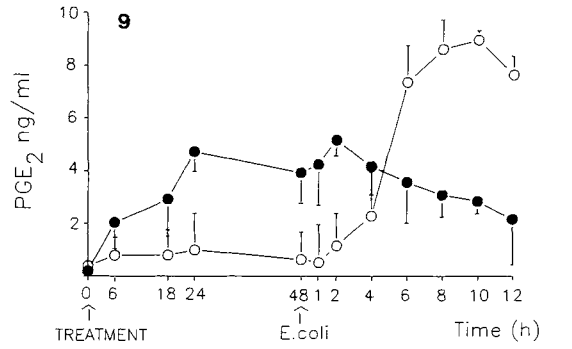


Fig. 4. Effect of GDM (filled circles) and saline (open circles) on levels of total peritoneal leucocytes prior to and following *E. coli* challenge. The results are expressed as mean ± SD.

Fig. 5. Bacteria in peritoneal fluid after pretreatment with GDM (filled circles) and saline (open circles). For experimental details, see Materials and Methods. The results are expressed as mean ± SD.

Fig. 6. Release of TNF (crosshatched columns), IL-1 (hatched diagonally columns), and PGE_2 (open columns) into medium from mouse peritoneal macrophages pretreated in vitro for 24 h with saline and GDM. The results are expressed as mean ± SD.

Fig. 7. Levels of IL-1 in peritoneal fluid prior to and following *E. coli* challenge after pretreatment with β-1,3-D-polyglucose derivatized microbeads (GDM) (filled circles) and saline (open squares). The results are expressed as mean ± SD.

Fig. 8. Levels of TNF in peritoneal fluid prior to and following *E. coli* challenge after pretreatment with β-1,3-D-polyglucose derivatized microbeads (GDM) (filled circles) and saline (open squares). The results are expressed as mean ± SD.

Fig. 9. Levels of PGE_2 in peritoneal fluid prior to and following *E. coli* challenge after pretreatment with β-1,3-D-polyglucose derivatized microbeads (GDM) (filled circles) and saline (open squares). The results are expressed as mean ± SD.

increased number of mononuclear cells could be seen after 6 h (data not shown). Animals given saline showed significantly decreased peritoneal leucocyte numbers following the injection.

One hour after challenge with live *E. coli,* both β-1,3-D-polyglucose treated and control groups showed significantly decreased numbers of total peritoneal cells. However, in animals treated with GDM the number of peritoneal leucocytes remained at a level 2–3 times higher than that of untreated animals. There was no significant difference on levels of total peritoneal leucocytes in animals pretreated with underivatized microbeads and animals given saline (data not shown).

There was no difference in the total levels of peritoneal leucocytes or differential counts in animals treated with GDM preincubated with polymyxin B (data not shown). The effect of β-1,3-D-polyglucose derivatives, as determined by their effect on peritoneal leucocyte numbers, was in principle identical in C_3H/Hej mice (data not shown).

Numbers of *E. coli* in Peritoneal Fluid

The number of bacteria in the peritoneal fluid of animals treated with GDM decreased gradually and approached zero after 24 h (Fig. 5). In untreated animals, there was a gradual increase in bacterial counts until the animals died about 12 h after challenge. Animals pretreated with GDM showed no signs of distress, and all of them survived several weeks past the experimental period.

Release of TNF, IL-1, and PGE_2 From Mouse Peritoneal Macrophages Pretreated With β-1,3-D-polyglucose Derivatives

There was no significant increase in the release of TNF into the medium from mouse peritoneal macrophages pretreated with β-1,3-D-polyglucose derivatives for 24 h compared with control cells (Fig. 6). Cells given β-1,3-D-polyglucose derivatives released about three times as much IL-1 into the medium as control cells. Cells pretreated with β-1,3-D-polyglucose derivatives released about four times more PGE_2 into the medium than control cells.

Peritoneal Fluid IL-1 Levels Following Treatment With β-1,3-D-polyglucose Derivatives and Subsequent Challenge With *E. coli*

The amount of IL-1 in peritoneal fluid increased significantly after 6 h and remained elevated until 48 h in GDM treated animals (Fig. 7). After challenge with *E. coli* there was again a slight increase in IL-1 levels, which declined to normal levels 12 h after challenge. Animals pretreated with saline did not have a significant increase in IL-1 during the following 48 h. Following challenge with *E. coli,* there was in these animals a pronounced increase in IL-1 levels with peak activity after 2 h. Twelve hours after challenge, just before the animals died of the infection, the levels of IL-1 had declined to about twice that of the GDM treated animals. There was no significant difference in levels of IL-1 in animals pretreated with underivatized microbeads compared with animals given saline (data not shown).

Peritoneal Fluid TNF Levels Following Treatment With β-1,3-D-polyglucose Derivatives and Subsequent Challenge With *E. coli*

The amount of peritoneal fluid TNF increased slightly after 6 h and declined to normal after 48 h in GDM treated animals (Fig. 8). After challenge with *E. coli* there was again an increase in TNF levels, which declined to normal 12 h after challenge. Animals pretreated with saline did not have a significant increase in TNF during the following 48 h. Following challenge with *E. coli,* in these animals there was a pronounced increase in TNF levels with peak activity after 1 h. Twelve hours after challenge, the levels of TNF had declined to normal in all groups. There was no significant difference in levels of TNF in animals pretreated with underivatized microbeads compared with animals given saline (data not shown).

Peritoneal Fluid PGE_2 Levels Following Treatment With β-1,3-D-polyglucose Derivatives and Subsequent Challenge With *E. coli*

The amount of PGE_2 in peritoneal fluid increased significantly after 6 h and remained elevated until 48 h in GDM treated animals (Fig. 9). After challenge with *E. coli* there was again a slight increase in PGE_2 levels, which remained elevated 12 h after challenge. Animals pretreated with saline did not have a significant increase in PGE_2 during the following 48 h. Following challenge with *E. coli* in these animals there was a pronounced increase in PGE_2 levels, with peak activity after 10 h. At the end of the experimental period the PGE_2 levels in untreated animals were increased three times compared with animals treated with GDM. There

was no significant difference in levels of PGE_2 in animals pretreated with underivatized microbeads compared with animals given saline (data not shown).

DISCUSSION

It is established that arachidonic acid metabolites inhibit the production of both IL-1 and TNF [22,23]. This agrees with our results. We have shown that macrophages stimulated in vivo with GDM release increased amounts of IL-1 and prostaglandin E_2 (PGE_2). We believe that the effects of macrophage secretory products largely explain the protection of β-1,3-D-polyglucose derivatives against the toxic effects of systemic infection. Our findings demonstrate that β-1,3-D-polyglucose affects mononuclear phagocytes partly through release of cytokines from macrophages and partly through other cellular events independent of identifiable substances released from pretreated mononuclear phagocytes. The cellular mechanisms underlying the enhanced bactericidal capacity are not well understood, however. Neutrophils stimulated with β-1,3-D-polyglucose derivatives do not produce increased amounts of oxygen radicals (C. Nathan, unpublished data) and do not produce increased amounts of lactoferrin (L.-T. Rasmussen, unpublished data). Mononuclear phagocytes stimulated with β-1,3-D-polyglucose derivatives do not produce enhanced amounts of IFN-gamma (E. Havell, unpublished data). We have preliminary observations indicating increased complement production in mononuclear phagocytes after treatment with β-1,3-D-polyglucose derivatives (L.-T. Rasmussen, unpublished data), but this can hardly account for the enhanced killing of bacteria. Our experiments suggest that the production of PGE_2 after administration of GDM is necessary for down regulation of lethal IL-1 and TNF activities (Figs. 7, 8, 9). Pretreatment of the animals with indomethacin, which inhibits release of PGE_2, resulted in "toxic" levels of the cytokines after challenge with *E. coli* and death of the animals within 12 h (data not shown). It remains to be investigated whether or not other immunologically relevant effects of IL-1 and TNF are also inhibited by indomethacin. Wallach et al. have shown that treatment with sublethal doses of IL-1 and TNF resulted in desensitization of mice to lethal effects of these cytokines [24]. Each of the two cytokines induced hyporesponsiveness to its own lethal effect, as well as to that of the other and to endotoxin. These findings suggest that some of the deleterious effects of TNF and IL-1 are modulated by antagonistic mechanisms. It has been shown that IL-1 induces effective and rapid decrease in TNF receptors, even though it binds to different and independent receptors. This is readily reversible, so that within a few hours of removal of IL-1 from pretreated cells, their TNF receptors were found to be fully restored. The desensitization to lethal effects of IL-1 and TNF, however, is maintained for at least 24 h. This indicates more slowly reversible changes of an as yet unclear nature. Our data indicate that desensitization may reflect an induced refractory state in cells which respond to TNF and IL-1 by production of some other mediators of the inflammatory response, e.g., leukotriens and prostaglandins. The effects of these mediators above a critical concentration may be the actual cause of death. But pretreatment with β-1,3-D-polyglucose derivatives induce a lower increase of cytokines after challenge with *E. coli* which in turn results in a lower increase in prostaglandins and leukotriens compared with controls. A decrease in responsiveness of mononuclear phagocytes to the effect of endotoxin may be caused by accumulation of immature, unresponsive macrophages and also by desensitization of mature macrophages. This unresponsiveness is reflected in decreased inducibility of effector cytokines of mononuclear phagocytes, including IL-1 and TNF. Consequently, pretreatment with GDM results in endotoxin tolerance which involves desensitization on the level of the formation of IL-1 and TNF and desensitization on the level of the effector function of these cytokines.

Czuprynski and Brown [25] have shown that intraperitoneal injection of small amounts of purified human or recombinant murine IL-1 resulted in an increased influx of leucocytes into the peritoneal cavity that peaked at 4–14 h after IL-1 injection. However, the time course of leucocyte accumulation was more rapid and declined to physiological state after 12–24 h. A single intraperitoneal injection of IL-1 is rapidly cleared from the circulation, but by administering β-1,3-D-polyglucose derivatives or other substances that enhance the autogenous production of IL-1 in a small and prolonged fashion, the increased levels of IL-1 are sustained both in peritoneal fluid and peripheral blood. Consequently, there are sustained enhancements of leucocytes in these compartments. Experiments performed with different immunomodulators

[26] showed that the duration of neutrophil leucocyte response can be classified into two types: a declining type, such as with IL-1, and a persistent type, such as with β-1,3-D-polyglucose. Since the general host response to foreign substances (e.g., lentinan, which is a β-1,3-D-polyglucose with glucopyranoside branching) is known to be of the declining type [26,27], the persistence of elevated levels of leucocytes with injection of β-1,3-D-polyglucose without branching seems to be a characteristic of the leucocyte inducing activity. Consequently, the chemical structure of polysaccharides seems to be related to the duration of the leucocyte response.

Our finding that a substantial portion of the β-1,3-D-polyglucose derivatives could be recovered in the milky spots of the omentum appears to highlight the significance of these organs in the resistance against peritoneal infection. The milky spots, first described by von Recklinghausen [28], are aggregates of lymphoid cells, notably macrophages, adjacent to the peritoneal surface and only partly covered by mesothelium [29]. Ranvier and others [30] speculated that peritoneal macrophages pass back and forth between the lymphoid tissue of the spots and the fluid of the peritoneal cavity. Our observations indicate that the number of macrophages in the milky spots can increase markedly in an inflammatory condition (Rasmussen et al., in preparation). The specific functions of macrophages in this location are not known, however. Since macrophage secretory products are instrumental in the protective effect of β-1,3-D-polyglucose derivatives [7,31], an investigation of the secretory capacity of the cells of the milky spots would seem pertinent.

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