Influence of Polyclonal Immunoglobulins on the Polymorphonuclear Leukocyte Response to Lipopolysaccharide of Salmonella enteritidis as Measured with Luminol-Enhanced Chemiluminescence

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In gram-negative sepsis, the activation of polymorphonuclear leukocytes (PMN) by lipopolysaccharide (LPS) and the resulting production of superoxide and other oxygen radicals may be an important cause of tissue damage. A suppression of the PMN response to LPS stimulation would be therapeutically beneficial. The aim of this study was to determine whether different polyclonal immunoglobulins (Igs; 5S-Ig, 7S-Ig, and 19S-Ig) influence the PMN response to LPS of Salmonella enteritidis in vitro. The respiratory burst activity of PMN was measured with luminol-enhanced chemiluminescence. After addition of a 5S-Ig solution containing F(ab′)2 fragments of IgG and a 19S-Ig solution containing 12% polyclonal IgM, luminol-enhanced chemiluminescence was reduced by 27% (P < 0.05) and 46% (P < 0.005), respectively. However, after addition of a 7S-Ig solution containing polyclonal IgG, luminol-enhanced chemiluminescence was increased fourfold (P < 0.05). The results suggest that the influence of polyclonal Igs on PMN response to LPS stimulation is dependent on the Ig class, F(ab′)2 fragments of IgG and IgM leading to LPS neutralization and IgG leading to the production of potentially toxic oxygen radicals.

The treatment of gram-negative sepsis with polyclonal immunoglobulins (Igs) is still the subject of controversy because of inconsistent data from clinical studies (29, 36). Even with broad-spectrum antibiotics and optimal supportive care, gram-negative sepsis remains a major clinical problem, especially in immunocompromised patients (10, 11, 37, 38). The tissue damage and the shock syndrome in gram-negative sepsis are due to a cascade of events, in which endotoxin or lipopolysaccharide (LPS), macrophages, polymorphonuclear leukocytes (PMN), and different mediators play a central part (25, 26, 35). LPS is a component of the outer membrane of gram-negative bacteria and is composed of several variable polysaccharide side chains (O antigen), a central oligosaccharide (core), and the biologically active lipid A (31).

LPS can directly activate macrophages and PMN or prime both for subsequent activation (41, 44). Macrophages respond primarily to the LPS stimulus with synthesis of tumor necrosis factor (TNF), interleukin 1, and platelet-activating factor. TNF is responsible for the shock syndrome in gram-negative sepsis (6, 7). After priming with LPS, macrophages can respond with production of leukotrienes and prostaglandins. PMN respond to LPS with a respiratory burst (20). During the respiratory burst of PMN, superoxide and other oxygen radicals (hydrogen peroxide, hydroxyl radical, singlet oxygen, and hypochlorous acid) are produced (2–4). Superoxide and the other oxygen radicals are responsible for tissue damage in gram-negative sepsis (5, 9, 32, 39). The oxidizing products of the respiratory burst can be reliably measured with luminol-enhanced chemiluminescence, the oxidation of the chemiluminescent luminol being accompanied by a striking emission of light (1, 21, 27).

The aim of this study was to investigate in vitro with luminol-enhanced chemiluminescence the influence of different Igs (5S-Ig, 7S-Ig, and 19S-Ig) on the PMN response to LPS stimulation.

MATERIALS AND METHODS

Reagents. LPS from Salmonella enteritidis (s-form; lot 113F-4005) and phorbol myristate acetate (PMA) were purchased from Sigma (St. Louis, Mo.). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Dextran T-250 and Luminol (5-aminoo-2,3-dihydro-1,4-phthalazine-dione) were obtained from Carl Roth (Karlsruhe, Germany). Luminol was dissolved in dimethyl sulfoxide. Human serum albumin was from Behring (Marburg, Germany). All other chemicals were analytical grade.

Polyclonal Igs. A pepsin-treated 5S-Ig solution (Gammavenin) in which the Fc region of Igs is removed after treatment with pepsin, leaving the active antigen-binding sites unaltered, was obtained from Behring. The 5S-Ig solution was composed of 70% shortened IgG (molecular weight, 95,000 to 105,000), 20% unaltered IgG (molecular weight, 150,000), and 10% Ig fragments. A 7S-Ig (Intraglobin) and a 19S-Ig (Pentaglobin) solution were obtained from Biotech (Frankfurt, Germany). The 7S-Ig solution contained 90% IgG (58% IgG1, 37% IgG2, 1.5% IgG3, and 4% IgG4) and IgM and IgA. The 19S-Ig solution contained 12% IgM (molecular weight, 900,000), 12% IgA, and 76% IgG. The content in different classes of Ig was determined by enzyme-linked immunosorbent assay (ELISA). The 7S-Ig and the 19S-Ig solutions were treated with beta-propiolactone during stabilization. For all the preparations used, the presence of <5% Igs in the form of polymeric aggregates was certified by the manufacturer.

Neutrophil isolation. Human PMN were isolated from blood samples form healthy volunteers by dextran sedimentation and Ficoll-Paque separation as described by Boyum (8). Heparin (12.5 U/ml) was used as an anticoagulant. Neutrophils were washed (200 × g, 10 min) in cell culture medium (MEM-Cl Dulbecco) and resuspended to a final concentration of 5 × 10⁹/ml in sterile phosphate-buffered saline (PBS).
(Dulbecco, calcium and magnesium free). The viability of PMN was 95% ± 5%.

Incubations. With sterile pipettes, the following substances were added to polystyrene cuvettes and incubated at 37°C in a shaking water-bath: (i) 100 µl of PMN cell suspension at 1.25 × 10⁶ cells per ml, (ii) 100 µl of luminol (10⁻⁵ M), (iii) 50 µl of heparinized donor plasma, with PBS as a control, and (iv) 50 µl of Ig solution (5S-Ig, 7S-Ig, or 19S-Ig), with human serum albumin as a control. Cells were incubated for 60 min at 37°C to eliminate stimulation through the isolation procedure. In preliminary experiments, it had been verified that neither human serum albumin nor IgGs had a stimulatory effect on PMN in the absence of LPS. Therefore, human serum albumin and IgGs were added prior to the preincubation. After 60 min of preincubation, the stimulus was added at time zero. The stimulus consisted of 100 µl of a solution of S. enteritidis LPS or PMA and PBS as positive and negative controls, respectively. The readings were started immediately after addition of the stimulus and registered every 5 min for 60 min.

Respiratory burst activity. At the times indicated, chemiluminescence was measured in a Lumac-Counter luminometer (Lumac Systems, Basel, Switzerland). The chemiluminescence was recorded in relative light units.

Statistics. Data are expressed as means ± standard errors of the mean for duplicate determinations of three similar experiments. The significance of differences was examined by use of an unpaired Student’s t test; P < 0.05 was considered significant.

RESULTS

Stimulation of PMN with PMA. The time course of chemiluminescence in PMN after addition of PMA (10 µg/ml), PBS, and LPS (2.5 µg/ml) is depicted in Fig. 1. The response to PMA was used to measure the ability of PMN to produce a respiratory burst. Ten minutes after the addition of PMA, a maximal response was obtained. The chemiluminescence declined slowly over the following 50 min. The potent stimulation of PMN by PMA was not significantly affected by the addition of IgGs. A similar response of the PMN was recorded after addition of formylmethionylleucylphenylalanine and zymosan (data not shown). In the presence of PBS, chemiluminescence did not exceed 200 relative light units. After addition of LPS (2.5 µg/ml), chemiluminescence increased rapidly to a peak after 15 min and declined progressively thereafter.

Stimulation of PMN with LPS. The dose-response curve for the stimulation of PMN with S. enteritidis LPS is shown in Fig. 2. The optimal luminol concentration, 10⁻⁵ M, had been determined in preliminary studies. A measurable stimulation of the PMN, as detected with the chemiluminescence assay, was seen with LPS concentrations ranging from 2.5 µg/ml to 2.5 ng/ml. The effect was dose dependent. Maximal stimulation was obtained 15 min after addition of LPS at 2.5 µg/ml. As stated above, chemiluminescence declined over the following 45 min. When donor plasma was replaced by human serum albumin and when donor plasma was heated for 30 min at 56°C, no stimulation of PMN with LPS was registered (data not shown). This indicates that the activation of PMN in the presence of LPS is complement dependent.

Effect of 5S-Ig. 5S-IgGs are G class IgGs in which the Fc region is removed by pepsin treatment. Hence, the activation of the complement system and the activation of PMN through the Fc receptor is suppressed, while the antigen binding remains unchanged. The treatment of PMN with a 5S-Ig solution at a concentration of 6.25 mg/ml prior to stimulation with LPS at 2.5 µg/ml resulted in a 27% decrease in chemiluminescence (P < 0.05) (Fig. 3). The kinetics are shown in Fig. 4A. The steep rise in chemiluminescence obtained with LPS at 2.5 µg/ml was suppressed after addition of 5S-IgGs. With lower concentrations of LPS (data not shown) or 5S-Ig concentrations of 625 or 62.5 µg/ml, the decrease in chemiluminescence was not significant (Fig. 4A). It had been determined in preliminary studies that the 5S-IgGs used had no effect on PMN in the absence of LPS.

Effect of 7S-Ig. 7S-IgGs are G class IgGs in which the Fc and Fab regions are unaltered. The treatment of PMN with a 7S-Ig solution at a concentration of 6.25 mg/ml prior to stimulation with LPS at 2.5 µg/ml resulted in a significant (ca. fourfold) increase in chemiluminescence (P < 0.05) (Fig. 3). The kinetics are shown in Fig. 4B. The peak chemiluminescence was recorded after 30 min, and the decrease was not complete, chemiluminescence being still high above LPS values after 60 min. When 7S-IgGs were added to PMN in the absence of LPS, no activation of PMN was recorded. With LPS concentrations of 250 or 25 ng/ml (data not shown) and with 7S-Ig concentrations of 625 or 62.5 µg/ml (Fig. 4B), the increase was not significant.
Effect of 19S-Igs. The 19S-Ig solution contained 12% polyclonal IgM and 76% IgG. Treatment of PMN with a 19S-Ig solution at a concentration of 6.25 mg/ml prior to stimulation with LPS at 2.5 μg/ml reduced the chemiluminescence by 46% (P < 0.005) (Fig. 3). The kinetics are shown in Fig. 4C. The effect was seen over the registered period. Nevertheless, the chemiluminescence increased slowly during the second half of the experiment. Again, the effect was not significant with lower concentrations of LPS (data not shown) or 19S-Igs (Fig. 4C).

DISCUSSION

Considering the LPS concentrations in our in vitro model, some restrictions have to be observed before extrapolating the results to clinical practice. The LPS concentrations used, 2.5 μg/ml to 2.5 ng/ml, were higher than found in bacteremia with the Limulus amebocyte lysate assay. However, concentrations were similar to those employed by other investigators for nonlethal bolus injections or continuous infusions of LPS in animals (9, 24, 34). In our study, the neutrophils were also stimulated with lower LPS concentrations, 250 and 25 ng/ml, but to a highly variable extent. The concentrations of Iggs used in the study were comparable to those used under clinical conditions. The sensitivity of our chemiluminescence assay and the kinetics obtained with PMA and LPS were comparable to those found in the literature (15, 28, 43). Complement-dependent stimulation of PMN by LPS was indicated by the fact that with human serum albumin or with heated donor plasma replacing donor plasma in the assay, no relevant increase in chemiluminescence was recorded. This is consistent with the theory that smooth-form LPS, like the S. enteritidis LPS chosen, requires the activation of the complement system via the alternative pathway before stimulating PMN (14, 45).

Treatment of the PMN with 5S-Igs before stimulation with LPS resulted in a 27% reduction in chemiluminescence. 5S-Igs are Ig molecules split by the proteolytic enzyme pepsin so as to remove the Fc portion and leave the two antigen-binding arms (Fab) connected, the bivalent portion being called F(ab′)2. Therefore, complement and PMN should not be activated via the Fc portion. We conclude that the reduction in neutrophil oxygen burst by 5S-Igs may due to a neutralization of the LPS stimulus by the F(ab′)2 fragments. The mechanisms of neutral-

FIG. 3. Luminol-enhanced chemiluminescence in human neutrophils recorded over a 60-min period after addition of LPS (2.5 μg/ml) at time zero: effect of pretreatment of neutrophils with 5S-Igs containing F(ab′)2 fragments, 7S-Igs containing polyclonal IgG, and 19S-Igs containing 12% IgM. Igs were added at a concentration of 6.25 mg/ml 60 min prior to the LPS stimulus. Data are means ± standard errors of the mean for duplicate determinations of three experiments. *, P < 0.05; **, P < 0.005.

FIG. 4. Luminol-enhanced chemiluminescence in human neutrophils: dose-response effect of pretreatment of neutrophils with different Iggs. The time course of chemiluminescence in the presence of 5S-Igs containing F(ab′)2 fragments (A), 7S-Igs containing polyclonal IgG (B), and 19S-Igs containing 12% IgM (C) was determined. Iggs were added 60 min prior to the LPS stimulus, which was added at time zero. Data are means for duplicate determinations of three experiments.
ization or detoxification are not fully understood, but it has been proposed by Ziegler et al. that antibodies bind to the LPS core, blocking access to lipid A (46). The exact epitope of the LPS core has been characterized for some chemically defined LPS (16).

The mechanisms of detoxification have been investigated by Shimosato et al., who first described the suppressive effect of human Igs on TNF production by rabbit macrophages stimulated with LPS (33). By analogy, it has been shown by several authors that the LPS-lipoprotein complex is much less active than free LPS in inducing the release of TNF, interleukin-6, and interleukin-1 from macrophages (13, 17, 42).

However, with 7S-Ig pretreatment of the PMN, the chemiluminescence was increased fourfold. Unlike 5S-Igs, 7S-Igs are unaltered IgG molecules containing both the Fab and the Fc region. Neutrophils, like many other cells, have membrane receptors for the Fc portion (18, 23). The increased activity of neutrophils in the presence of 7S-Igs may be explained by a direct stimulation via the Fc receptor. Moreover, the increased activity of neutrophils was largely LPS dependent. In the absence of LPS, PMN were not stimulated by Igs, in particular, by 7S-Igs. The reason may be that the Fc receptor binds IgG molecules much more efficiently if antigen is bound to the IgG molecules, probably due to a configurational change in the Fc region (40). It should be noted that stimulation of the neutrophils may be detrimental in the clinical setting, where superoxide anion production may lead to tissue damage (5, 9, 33, 39).

A 46% reduction in chemiluminescence was obtained with 19S-Ig pretreatment of the PMN. As has been postulated for the 5S-Igs, the decreased activity of the neutrophils in the presence of 19S-Igs may be explained by a neutralization of the LPS stimulus. The more pronounced effect is possibly due to the enrichment with IgM: the 19S-Igs used contained 12% IgM molecules. IgM molecules may be more effective in neutralizing LPS. These findings are consistent with the results of a recent clinical trial which demonstrated that HA-1A, a monoclonal IgM antibody against lipid A, significantly reduces mortality in patients with sepsis and gram-negative bacteremia (37). That a reduction in chemiluminescence was recorded in the presence of 76% IgG may be explained by the fact that the neutralizing effect of IgM is more effective than or even prevents the stimulating effect of IgG.

Taken together, our data suggest that the composition of the polyclonal Igs [F(ab')2], fragments, unaltered IgG, or IgM largely influence their effect on the PMN response to LPS stimulation. Since the preparations used in this study have not been characterized with respect to the binding capacity for S. enteritidis LPS, it cannot be excluded that differences between the various preparations in the ability to bind LPS affected the PMN response. However, major differences seem unlikely in view of the very similar binding capacity of polyclonal IgG for numerous antigens tested by the manufacturers. Furthermore, the disparity between preparations obtained from the plasma of more than 1,000 donors should be minimal. Thus, it appears that the variable composition of the polyclonal Igs may be one reason for the equivocal results of clinical studies, i.e., with antibodies to J5 (12, 47), and should be respected in further trials.

REFERENCES