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Protective capacity of a IgM/IgA-enriched polyclonal immunoglobulin-G preparation in endotoxemia

Steffen Oesser, Claas Schulze, Jürgen Seifert

Surgical Research for the Department of General Surgery and Thoracic Surgery, University of Kiel, Kiel, Germany

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Abstract. Animal experiments were carried out to investigate whether a protective effect can be achieved in endotoxemia by intravenous (i.v.) application of a polyclonal immunoglobulin preparation (IVIG-IgG/A/M) enriched with 12% IgM and 12% IgA. Following administration of IVIG-IgG/A/M (500 mg/kg), endotoxemia was induced by intraperitoneal inoculation of a sublethal dose (5×10^8 CFU/kg) of Escherichia coli (*E. coli*) and subsequent i.v. administration of an antimicrobial agent (Imipenem). Plasma endotoxin activity, IL-6 activity, mean arterial pressure, and skeletal muscle oxygen pressure (tpO_2) were measured at regular intervals over a total observation period of 7 h. Prophylactic administration of IVIG-IgG/A/M was found to significantly attenuate ($P<0.01$) the antibiotic-induced increase in endotoxin activity as compared to the albumin control group. Limited endotoxemia in the IgG/A/M group was associated with reduced levels of circulating IL-6 ($P<0.01$). Both lipopolysaccharide-induced hypotension and depression of tissue oxygenation were attenuated ($P<0.01$) by pre-treatment with IVIG-IgG/A/M. The experimental results suggest that in endotoxemia the polyclonal immunoglobulin preparation has a prophylactic protective effect on the acute phase responses and reduces the cardiodepressant effects of *E. coli* septicaemia.

Key words: IVIG – Immunoglobulin – Endotoxemia – Sepsis

Introduction

The therapeutic value of passive immunization with polyclonal immunoglobulin preparations in serious gram-negative infections is still a matter of controversy. This contributes to the lack of convincing, quantitative data on the degree and scope of the protective capacity of immunoglobulin preparations in sepsis syndrome. In spite of the development of more and more potent anti-

Correspondece to: S. Oesser; Tel.: +49-431-5971966, Fax: +49-431-5971956

microbial agents, the mortality rate in patients with serious gram-negative infections is still 20–30% [2, 37]. The development of septic shock increases the fatality rate to 43–80% [46, 47].

It is well accepted that many of the pathophysiological sequelae observed during serious gram-negative bacterial infections are related to the effects of endotoxin (lipopolysaccharide [LPS]) [13], an integral component of the outer membrane of gram-negative bacteria, upon the mammalian host. LPS provokes the release of a series of so-called secondary mediators that appear to be responsible for the deleterious effects. Depending on their mode of action, antibiotics induce more or less endotoxin release in gram-negative infections [18, 33]. The concentration of endotoxins in the plasma as well as the duration of endotoxemia both correlate closely with development of septic organ failure [9, 15]. For this reason, the therapeutic concept for septic patients should cover not only removal of the focus, antimicrobial therapy and reduction of the effects of mediators, but should also employ means to reduce endotoxicity [12, 34, 38].

In addition to cross-reactive monoclonal antibodies that bind to common antigens on LPS [35, 48], polyclonal antibody preparations have also been used in therapy of sepsis as hyperimmune serum [16, 47] and as immunoglobulin preparations [19, 27, 41].

Positive initial clinical results [24, 25, 29, 48] indicating that prognosis of septic organ failure might be improved significantly by the administration of monoclonal antibodies directed against the deep core region of LPS were not supported by subsequent studies [5, 30]. Failure of the monoclonal antibodies HA-1A and E5 aroused the suspicion that these reagents may not effectively bind endotoxin or neutralize its harmful effects. Previously published experimental studies on the protective capacity of polyclonal antibody preparations show contradictory results [22, 40, 42]. Therefore, the aim of our experimental studies was to clarify the therapeutic value of IgM-enriched immunoglobulin preparations in endotoxemia with special regard to endotoxin- and IL-6 levels and bacterial count in blood.

In this study a commercially available polyclonal immunoglobulin preparation (IVIG-IgG/A/M) enriched with 12% IgM was used, which had already led to positive results in clinical trials [27, 36, 41]. An established rat intra-abdominal infection model was chosen to evaluate the protective capacity in endotoxemia of this immunoglobulin preparation under standardized conditions.

Materials and methods

Immunoglobulin

A polyclonal immunoglobulin preparation with 12% IgM was used (Pentaglobin 76% IgG, 12% IgM and 12% IgA, lot no.: 146044, BIOTEST-Pharma AG, Dreieich, Germany).

Albumin

A 5% human albumin solution, lot no.: 188011 (Behring AG, Marburg, Germany) was used.

Bacteria

An *E. coli* strain (ONT:H16) isolated from septic patient blood cultures was used in the experiments.

Animal model

The experiments were performed on male Wistar rats (body weights between 300 g and 350 g). Anesthesia was initiated with 90 mg ketamine/kg of b.w. i.p. (Ketanest, Parke-Davis) and continued with 25 mg ketamine/kg b.w. (i.m. every 30 min). A quantity of 0.6 ml of blood (35 IU heparin/ml) was taken from the jugular vein at the beginning of the experiment and 1, 3, 5, and 7 h later. The bacterial count was determined in the blood. Endotoxin and IL-6 activity were determined in the plasma. The animals were randomized in two groups. Immediately after the first blood sample, a 12% IgM-enriched immunoglobulin preparation (Pentaglobin, 500 mg/kg b.w.) was administered intravenously (i.v.) to one group of the animals (IgG/A/M group, n=13). A placebo group (n=13) received albumin (500 mg/kg b.w.).

Following i.v. administration of the protein solutions in both groups, a suspension of *E. coli*, 5×10^8 CFU/kg b.w., was administered intraperitoneally (i.p.). A further group of seven animals (control group) received no bacteria (0.9% NaCl instead), but instead received albumin as in the placebo group. One hour after i.p. bacterial challenge, all three groups were treated with 14 mg/kg b.w. imipenem (=28 mg/kg imipenem/cilastine = Zienam, MSD Munich, Germany) through the jugular vein.

Mean arterial pressure (MAP), measured in the cannulated left common carotid artery, was monitored continuously throughout the 7-h experiment.

Registration of skeletal muscle oxygen pressure

Mean skeletal muscle oxygen pressure (tpO_2) was continuously recorded throughout the 7-h experiment using a Licox 2-channel metering device (GMS, Kiel, Germany). For this purpose a modified Clark-type microcatheter electrode integrating local tpO_2 distribution over a range of 7 mm (GMK-pO_2-COS 200, GMS, Kiel, Germany) was implanted in the left triceps surae muscle. Recorded values were temperature-corrected by means of an Ni/-NiCr thermoelement placed in the contralateral triceps surae muscle of the animal.

Bacterial count

To determine the blood bacterial count, 100 µl of blood from each animal was incubated on blood agar plates for 24 h at 37°C.

Anti-LPS titer

LPS extraction of the *E. coli* strain used (ONT:H16) and determination of ELISA titers against these LPS and against LPS from *E. coli* O111:H^- were performed as described by Autenrieth et al. [3].

Determination of plasma endotoxin activity

Plasma endotoxin activity was determined by means of a modified LAL test using a chromogenic substrate [32]. After 1:40 dilution with 0.9 % NaCl and heating (80°C, 5 min) 100 µl of the solution was incubated with 50 µl Limulus lysate (Pyroquant 50, Associates of Cape Cod) for 45 min at 37°C. Then 100 µl chromogenic substrate (S-2423, Chromo-

genix, Mølndal, Sweden) was added and the mixture incubated for 4 min (37°C). Incubation was stopped by addition of 50 µl of glacial acetic acid. Optical density was measured at 405 nm. The sensitivity was 0.02 EU/ml.

The calibration curve was established by spiking the pooled plasma of healthy rats with *E. coli* endotoxin (EC-5).

Dose-dependency of inhibition of LAL-activity by IgG/A/M

To quantify a possible dose-dependency of the effect of IgG/A/M on endotoxin activity, the immunoglobulin preparation was administered to the animals in various concentrations (62.5 mg/kg, 250 mg/kg and 500 mg/kg b.w.). For these additional experiments a study design was used as described above. The observation period was limited to 5 h.

The percentage of endotoxin inactivated by different doses of IgG/A/M was compared to the percentage of endotoxin inactivation in the albumin placebo group 5 h after bacterial challenge.

Determination of IL-6 activity in plasma

IL-6 activity was determined by using an IL-6-dependent B9.9–3A4 cell proliferation assay [45]. The assay is performed in 96-well flat-bottom microtiter plates, whereby 2×10^3 cells were used in each well for a test volume of 200 µl. A quantity of 100 µl of culture medium (RPMI 1640, 10 % FCS, 5×10^{-5}M 2-mercaptoethanol) was added to the sample volume of 33 µl and diluted 1:4 in seven steps. A standard preparation of IL-6 standard giving 330 U/ml was used as a positive control. Following incubation (50 h, 37°C, 5% CO_2) 10 µl MTT solution (5 mg/ml in PBS, pH 7.4) was added to each well and the cells were incubated for a further 3 h. The MTT reaction was stopped by adding 100 µl of isopropanol. Optical density was then measured at 550 nm with a microplate reader (MR 700; Dynatech). IL-6 activity in the samples was determined by using a standard preparation of IL-6 (330 U/ml) followed by comparison of the dilutions, revealing a proliferation of 50 % by probit analysis [23]. The results are given in units of IL-6 activity per milliliter.

Statistics

All results are expressed as mean values ± SD. The significance of differences in values was assessed by the Mann-Whitney U-test and the unpaired Student's test (for influence of IgG/A/M dose). $P<0.05$ was considered statistically significant.

Results

Anti-LPS titer

In the lot of IgG/A/M used in these experiments, the anti-LPS ELISA titers against the LPS of the *E. coli* ONT:H16 were found to be in a similar range (1:1600) when compared with the ELISA titers against LPS from *E. coli* O111:H$^-$ used as a reference strain (1:800).

Blood bacterial count

Immediately following i.p. administration of 5×10^8 *E. coli* CFU/kg b.w. into the peritoneal cavity, the bacterial count in the blood began to rise and reached,

in the albumin group, approx. 1,800 CFU/ml after 1 h. At this point, the blood bacterial count in the IgG/A/M group (1,600 CFU/ml) was somewhat lower than in the albumin group, although the differences were not significant. In both groups, administration of the antibiotic led to a reduced blood bacterial count that was registered as early as 60 min later. Two hours after administration of the antibiotic, the blood bacterial counts were significantly lower ($P<0.05$) in the animals of the IgG/A/M group (102±70 CFU/ml) than in those of the albumin group (238±181 CFU/ml). Bacteria were detectable in the blood of both groups until the end of the experiment (7 h after i.p. bacterial challenge), whereby the differences between the two groups were no longer significant. No positive blood cultures were found in animals of the 0.9% NaCl control group, which had received no *E. coli*.

Inhibition of LAL activity in plasma

A gradual slight increase in plasma endotoxin activity could be detected 1 h after i.p. administration of the bacteria in both the albumin group and the IgG/A/M treated group (Fig. 1), which continued to increase until the end of the observation period.

Considerable differences were, however, found between plasma endotoxin activity in the two groups as early as 2 h after administration of the antibiotic (i.e. 3 h after the beginning of the experiment), which are highly significant ($P<0.01$). At this time, mean endotoxin activity in the IgG/A/M group was 2.11±0.68 EU/ml and 12.14±7.07 EU/ml in the albumin group. In the albumin group, plasma endotoxin activity rose continuously throughout the experiment, reaching 44.42±9.58 EU/ml 6 h after bacterial challenge (Fig. 1). At this point, endotoxin activity in the albumin group was approximately four times that found in the group treated with IgG/A/M (11.15±3.11 EU/ml).

In the control group animals, which were treated with albumin and an antibiotic but no *E. coli*, plasma endotoxin activity increased minimally 1 h after administration of the antibiotic (0.08±0.07 EU/ml) and reached levels of 0.11±0.07 EU/ml at the end of the observation period.

Dose dependency of IgG/A/M and LAL activity in plasma

To find out whether there was a correlation between plasma endotoxin inactivation and the IgG/A/M dose administered, the percentage of endotoxin inactivation was measured 5 h after i.v. IgG/A/M administration for different doses of the immunoglobulin preparation (6.25 mg/kg, 250 mg/kg, 500 mg/kg).

A comparison of the endotoxin inactivation percentages revealed significant differences between the various IgG/A/M doses ($P<0.01$). Increasing the IgG/A/M dose led to a decrease in plasma endotoxin activity. Mean inactivation is 41% (41±4%, n=8) at an IgG/A/M dose of 62.5 mg/kg b.w., 79% (79±6%, n=14) at 250 mg/kg b.w. and 87 % (87±3%, n=13) with an IgG/A/M dose of 500 mg/kg b.w.

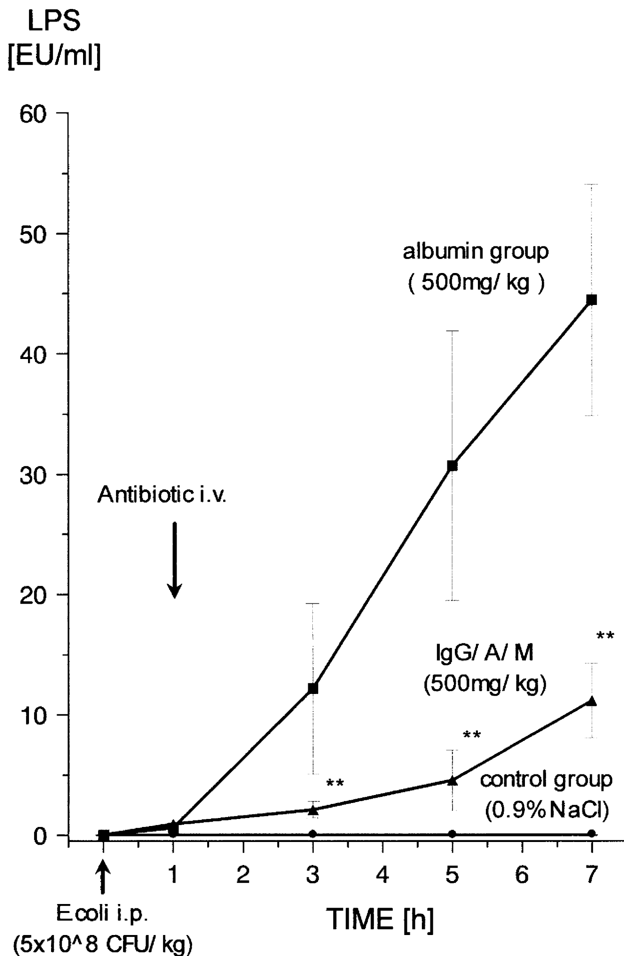

Fig. 1. Antibiotic induced endotoxemia in intraabdominal *E. coli* infection. Effect of i.v. immunoglobulin preparation (*IgG/A/M*) or i.v. albumin on plasma endotoxin activity in animal model. (Mean ± SD, n=13). The control group animals (n=7) received no *E. coli* (0.9% NaCl instead) but albumin, as in the placebo group (500 mg/kg). ** P<0.01 versus albumin group

Inhibition of LPS-induced IL-6 secretion

A significant increase in plasma IL-6 activity could be found after administration of the antibiotic. The increase in IL-6 activity was much greater in the albumin group than in the animals treated with IVIG-IgG/A/M (Fig. 2). Two hours after administration of the antibiotic, plasma IL-6 activity in the IgG/A/M group was significantly lower (P<0.01) than in the albumin group. This difference between the therapy and albumin groups remained until the end of the experiment (Fig. 2).

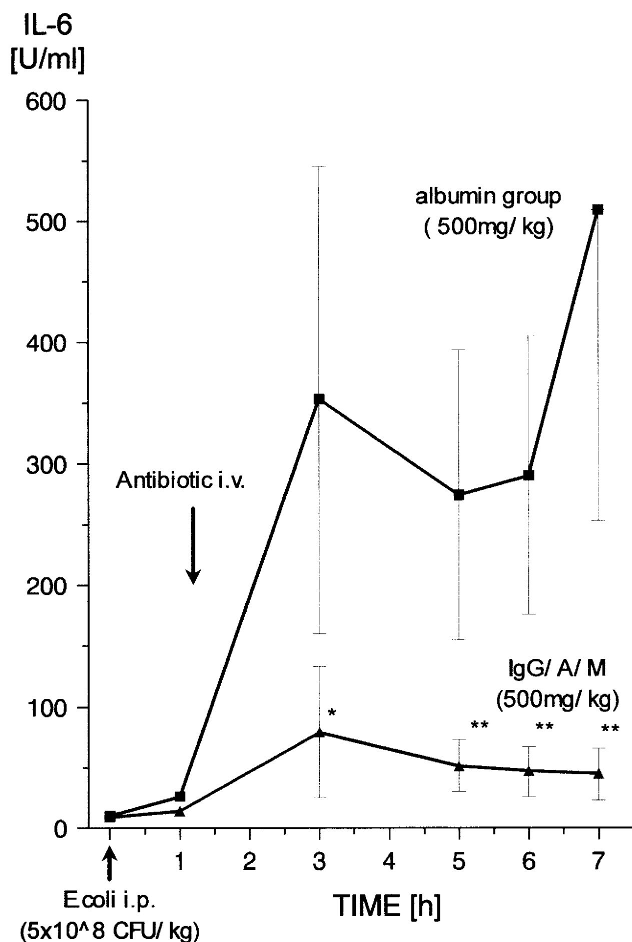

Fig. 2. Inhibition of lipopolysaccharide (LPS)-induced IL-6 secretion in antibiotic induced endotoxemia in rats by pretreatment with i.v. *IgG/A/M* (n=13) as compared to the albumin-group (n=13). (Mean ± SD). *P<0.05 versus albumin group; **P<0.01 versus albumin group

Inhibition of endotoxin-induced hypotension

A continuous decrease in MAP was observed in the albumin-group animals beginning 3 h after bacterial challenge, i.e. 2 h after i.v. administration of the antibiotic (Fig. 3). The resulting difference in MAP as compared to the control group was highly significant (P<0.01) at the end of the observation period. At this time the difference in MAP compared with values at the beginning of the experiment was 55 mm Hg in the albumin group.

A much more stable MAP was registered in the animals of the IgG/A/M group than in those of the albumin group. Four hours after antibiotic administration, MAP in the IgG/A/M group was significantly (P<0.05) higher than

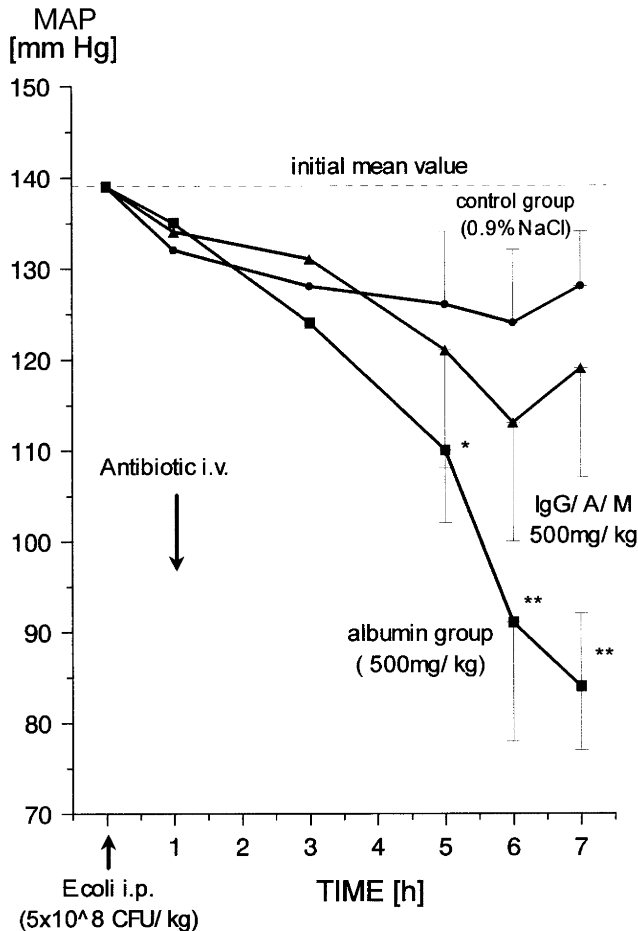

Fig. 3. Prevention of endotoxin-induced hypotension in rat model by i.v. administration of IgG/A/M. Mean arterial pressure (MAP) of the albumin group (n=13), the *IgG/A/M* group (n=13) and the 0.9% NaCl control group (n=7). (Mean ± SD). The control group animals received no *E. coli* (0.9% NaCl instead) but albumin, as in the placebo group (500 mg/kg). *P<0.05 versus IgG/A/M group; **P<0.01 versus IgG/A/M group

MAP in the albumin group. In the further course of the experiment, the differences in MAP between the two groups became even more pronounced and were highly significant (P<0.01) at the end of the experiment. For the IgG/A/M group, the mean difference from the initial value was only 21 mm Hg at this point (Fig. 3).

As was to be expected, only a slight decrease in MAP was registered in the control group animals. At the end of the experiment, the mean MAP decrease in this group did not exceed 15 mm Hg below the initial value.

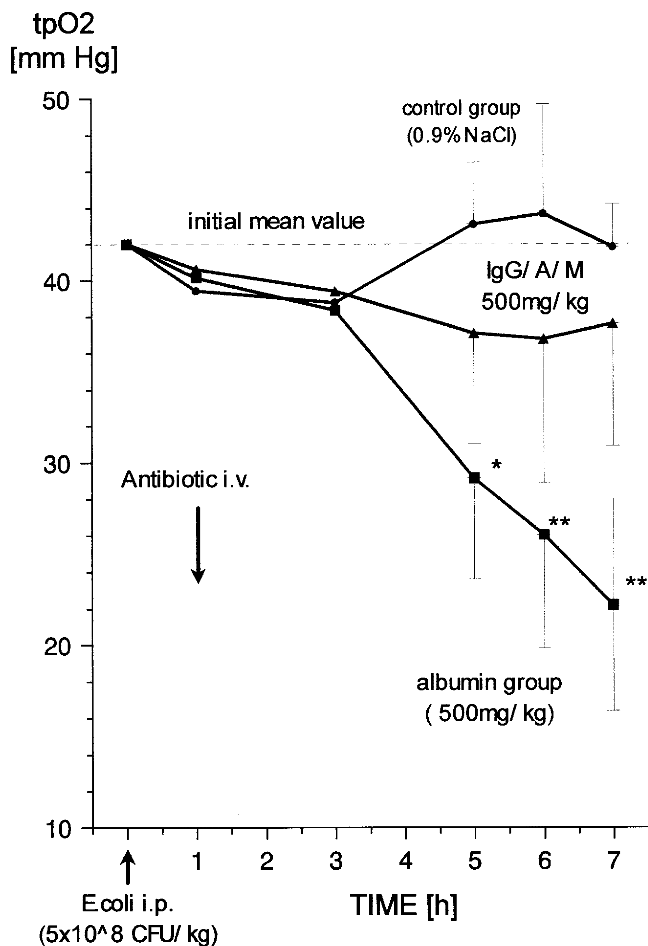

Fig. 4. Skeletal muscle oxygen pressure in endotoxemic rats. Influence of i.v. administration of *IgG/A/M* or albumin (Mean ± SD, n=12). The control group animals (n=6) received no *E. coli* (0.9% NaCl instead) but albumin, as in the placebo group (500 mg/kg). $*P<0.05$ versus IgG/A/M group; $**P<0.01$ versus IgG/A/M group

Inhibition of LPS-induced decrease of tpO_2

tpO_2 varied greatly among the animals in the three groups during the second half of the observation period. Muscle oxygen pressure in the control group (no bacterial challenge) had slightly increased approximately 3 h after the beginning of the experiment. The same parameter in the albumin placebo group had decreased 2 h after administration of the antibiotic. This decrease continued until the end of the observation period. The mean difference from the initial value was 18 mm Hg (Fig. 4).

The course of the skeletal muscle oxygen pressure could be compared with that of blood pressure in the IgG/A/M group. Muscle oxygen pressure in the

IgG/A/M group was much more stable than in the albumin placebo group. In the IgG/A/M group, this parameter was significantly higher ($P<0.05$) 4 h after administration of the antibiotic, when compared with the albumin group. At the end of the experiment, the tpO_2 differences between the two groups were highly significant ($P<0.01$).

Discussion

Since clinical studies [16, 41] have confirmed that therapy with polyclonal immunoglobulin preparations can be expected to have a beneficial effect in septicemia, these agents are accorded a certain significance within adjunctive concepts for the treatment of septicemia [8]. The benefit of adjunctive therapy of sepsis with polyclonal immunoglobulin preparations is still a matter of controversy [5, 6, 46]. One of the reasons for this contradictory situation may be that significant effects were not found after the administration of polyclonal immunoglobulin preparations in some clinical trials [11, 28]. In spite of improved scoring systems, clinical studies involving patients with sepsis syndrome in many cases still face the problem that the results are influenced to a considerable degree by the differences in septic status of patients when enrolled. The point in the course of sepsis at which adjuvant treatment is started has a significant influence on patient outcome [8].

Very few convincing experimental data have been published to prove the reduction of endotoxemia by polyclonal immunoglobulin preparations. Most experimental studies on the therapeutic relevance of immunoglobulin preparations address only partial aspects of the effects of immunoglobulin therapy, such as opsonic activity [22] or antipyretic activity [26]. The observation that no protective effect was achieved in endotoxin shock after i.v. administration of a polyclonal IgG preparation [40] cast considerable doubt on the efficacy of immunoglobulin therapy in endotoxemia.

Characteristics of an anti-endotoxin agent that would be desirable for therapeutic application include specific and avid binding of LPS concomitant with LPS neutralizing activity to prevent triggering of the lethal sepsis syndrome. Since the binding of the anti-LPS antibody to a certain region of LPS in vitro is not sufficient to predict the efficacy of these agents in vivo, an animal model was chosen to quantify the LPS-neutralizing capacity of polyclonal immunoglobulin preparations in which the endotoxemia originates from a gram-negative focus. In the experimental setting, endotoxin was not administered as a bolus, but a more continuous release of endotoxin was induced by administration of an antimicrobial agent. The object of these experiments was the quantification of the influence of an IgG/A/M preparation on endotoxin activity and endotoxin-associated pathophysiological changes rather than the influence on mortality rate. Therefore, a sublethal dose of bacteria was administered. Since preliminary experiments did not show clinically relevant effects in rats below a certain blood endotoxin activity level, the bacterial count selected for the injections was relatively high. Due to the resulting high endotoxin level, an increased dosage of polyclonal immunoglobulin preparation – above the recommended human dose – was selected for the experiment.

In the albumin group, a clear relation was seen between the plasma endotoxin activity levels measured in the LAL test and the secretion of IL-6 by macrophages. The changes in the clinical parameters observed in the albumin group such as the drop in mean arterial pressure and the decrease in tissue oxygen pressure correlated closely with the increase in endotoxin activity measured in the plasma. The drop in blood pressure observed in animals with high plasma endotoxin levels was due to a decrease in cardiac output seen in endotoxemia [1] and was caused by an increase in NO release, since endotoxin induces increased formation of NO from L-arginine[21].

Changes in tissue oxygen pressure in skeletal muscle are a very sensitive indicator of microcirculatory disorders leading to reduced oxygen supply. This parameter facilitates early and sensitive registration of microcirculatory disorders [7, 20]. The decreased tissue oxygen pressure concomitant with increasing plasma endotoxin activity reflects changes in both cardiac output and local shunting processes.

As seen in the experiments, prophylactic administration of polyclonal IVIG-IgG/A/M enriched with 12% IgM prevented a marked increase of endotoxin activity in plasma following i.v. application of the antibiotic. As an indicator of the protective capacity of IgG/A/M, the difference in plasma endotoxin activity between the IgG/A/M and albumin control groups was highly significant as early as 2 h after administration of the antibiotic. The reduction of plasma endotoxin activity by IgG/A/M was dose-dependent. Following administration of 500 mg/kg of IgG/A/M, the difference between the therapy and albumin groups reached a maximum of approximately 87% in this animal model.

Prophylactic i.v. administration of IgG/A/M not only prevented endotoxin-induced hypotension, as seen in the MAP of the IgG/A/M group at the end of the experiment, it also reduced endotoxin-induced microcirculatory disorders measured in skeletal muscle as reflected in tissue oxygen pressure.

Many of the pathophysiological responses in sepsis syndrome are known to be mediated by the network of cytokines [31].

In animal experiments as well as in clinical studies interleukin-6 seems to be a good marker of severity during bacterial infection and correlates well with outcome [4, 14, 39]. Because of its well-defined role in sepsis, IL-6 measurement was included in the experimental protocol of the present study.

As reflected by differences of IL-6 levels between the albumin and therapy groups, the release of potentially deleterious endogenous secondary mediators in response to *E. coli* LPS is markedly reduced by the polyclonal antibody preparation used in these experiments.

The relation observed in our experiments between reduction of endotoxin activity and IgG/A/M dose does not in itself provide a sufficient answer to the question of how the protective effect is achieved. Since antibody titers against the LPS from the *E. coli* strain used in the experiments were found to be relatively high in the IgG-fraction as well as in the IgM-fraction of the IgG/A/M lot used, it may be assumed that the protective effect of this IgG/A/M-preparation is achieved mainly by anti-LPS antibodies. Antibodies against LPS may be directed against one or more of the three principal regions of the LPS molecule: i.e. (1) the outermost O-antigen polysaccharide region unique to each particular strain of gram-negative bacteria, (2) the core polysaccharide region

similar in many strains of gram-negative bacteria that couples O-antigen polysaccharide to the (3) highly conserved lipid A region representing the portion of the LPS molecule responsible for toxicity. It has been proposed that anti-LPS antibodies may act by enhancing microbial killing, thereby preventing bacterial proliferation and endotoxin release, by neutralizing the toxic effects of LPS or by promoting LPS clearance from the systemic circulation.

The inhibitory activity of IgG/A/M in the LAL assay, on IL-6 release and on the other biological activities of LPS could be explained by some form of steric hindrance, disaggregation of supramolecular LPS structures, or by a modification of the conformation of the LPS molecule after binding of the antibody. No matter what hypothesis is used to explain the blockage of lipid A binding to the macrophage cell membrane by the antibodies of the IgG/A/M preparation, our animal experiments demonstrate that i.v. IgG/A/M with high antibody titer against causative human pathogenic organism is highly effective in neutralizing the pathophysiological effects of LPS.

The significantly lower bacterial counts in the IgG/A/M group found 2 h after administration of the antibiotic reflects the antibacterial activity of the polyclonal immunoglobulin preparation in addition to the endotoxin-binding effect. The synergistic effect postulated for combined administration of polyclonal immunoglobulin preparations with an antibiotic [17] is confirmed by these results.

Some authors assume that the antibodies of the IgM fraction provide more protective capacity than those of the IgG fraction [29, 43, 44]. Because of the higher opsonic activity of IgM antibodies, one can expect that IgM anti-LPS will enhance the complement-mediated internalization of LPS by macrophages better than will IgG anti-LPS [22, 35]. Fc-mediated and opsonin-mediated antibody interactions may play an important role in the protective activity of anti-LPS antibodies [10]. The lack of the IgM fraction might also explain the limited protection of i.v. IgG observed both in animal experiments [40] and in clinical trials [8]. This leads to the conclusion that the neutralization capacity of i.v. IgG possibly does not suffice to achieve rapid, i.e. therapeutically relevant, inactivation of the endotoxin. Experimental studies should be performed to elucidate further the possible influence of the immunoglobulin classes on the endotoxin-neutralizing capabilities of antibody preparations.

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