Differences in Innate Defense Mechanisms in Endotoxemia and Polymicrobial Septic Peritonitis

BERND ECHTENACHER, MARINA A. FREUDENBERG, ROBERT S. JACK, and DANIELA N. MÄNNEL

Max-Planck-Institute for Immunobiology, Freiburg, Institute for Immunology, University of Greifswald, Greifswald, and Institute for Pathology/Tumor Immunology, University of Regensburg, Regensburg, Germany

Received 8 June 2001/Returned for modification 13 July 2001/Accepted 20 August 2001

Loss, reduction, or enhancement of the ability to respond to bacterial lipopolysaccharide (LPS) has no influence on survival of mice in a model of postoperative polymicrobial septic peritonitis induced by cecal ligation and puncture (CLP). This was demonstrated by using either mice with a defective Tlr4 gene, which encodes the critical receptor molecule for LPS responses, or mice deficient for LPS binding protein (LBP) or mice sensitized to LPS by Propionibacterium acnes. Though interleukin-12 (IL-12) and gamma interferon (IFN-γ) play an important role in the sensitivity to LPS as well as in the resistance to several infections, loss of these cytokine pathways does not affect survival after CLP. Thus, neutralization of neither endogenous IL-12 nor IFN-γ altered mortality. In addition, IFN-γ receptor-deficient mice demonstrated the same sensitivity to CLP as mice with a functional IFN-γ receptor. However, administration of IFN-γ at the time of operation or pretreatment of both IFN-γ-sensitive and IFN-γ-resistant mice with IL-12 significantly enhanced mortality. This indicates that in the present infection model activation of innate defense mechanisms is not dependent on LPS recognition and does not require endogenous IL-12 or IFN-γ function. Indeed, exogenous application of these two mediators had deleterious effects.

During septic peritonitis gram-negative and gram-positive bacteria are transported via lymphatics and blood into vital organs. Lipopolysaccharide (LPS) is an important target molecule for recognition of gram-negative bacteria by the innate immune system and a widely used model substance which, depending on the dose, causes inflammation, shock, or death (7). LPS binding protein (LBP) is a plasma protein that accelerates binding of LPS to CD14 and thereby considerably enhances the host’s sensitivity to LPS (23). Thus, the host perceives minute concentrations of LPS, which normally indicate a bacterial infection, and can mount an antibacterial response while the infection is still at an early stage. Inactivation of the LBP gene reduces the susceptibility of mice to LPS and increases their susceptibility to a Salmonella enterica serovar Typhimurium infection (12). Similarly, mutation or deletion of the Tlr4 gene which results in LPS unresponsiveness (22, 27) leads to a high susceptibility to infection with S. enterica serovar Typhimurium (21, 29) and encapsulated Escherichia coli (2).

The cytokines interleukin-12 (IL-12) and gamma interferon (IFN-γ) are important mediators of innate immune reactions, and both are released after bacterial infection or challenge with LPS. In a number of bacterial infection models survival or clearance of pathogens either requires IFN-γ or IL-12 or is enhanced when these cytokines are administered exogenously. IL-12 neutralization impaired the clearance of intraperitoneally (i.p.) instilled E. coli (33) and inhibited resistance against Yersinia enterocolitica (1), whereas treatment with IL-12 before or after infection with streptococci increased survival (19).

Survival in a model of septic peritonitis (colon ascendens stent peritonitis [CASP]) required IFN-γ receptor (IFN-γR) activation (31), and IL-12 frequently exerts its protective effects through induction of IFN-γ (1, 32). Both cytokines, however, contribute to mortality after lethal LPS challenge, as demonstrated in mice pretreated with Propionibacterium acnes (5, 6) or infected with Mycobacterium bovis BCG (30). Recently, mice pretreated with P. acnes were shown to be highly sensitive to high-dose S. enterica serovar Typhimurium infection (M. Gumenscheimer and M. A. Freudenberg, unpublished data).

These findings raised the question of how LPS-insensitive (TLR4- and LBP-deficient) or LPS-hypersensitive (P. acnes-primed) mice would react in a more complex, clinically relevant model of septic peritonitis induced by cecal ligation and puncture (CLP). After CLP a postoperative, mixed, bacterial septic peritonitis, characterized by a septic focus and a protracted course of systemic infection, develops. The host becomes exposed to the whole range of intestinal flora, including gram-negative bacteria. Therefore, survival after CLP was expected to be improved by endogenous or exogenous IL-12 or IFN-γ. Surprisingly, however, we found that survival after CLP was not affected by loss of TLR4 or LBP, the presence of endogenous IL-12 or endogenous IFN-γ, or sensitization with P. acnes but was decreased by treatment with IL-12 or IFN-γ.

MATERIALS AND METHODS

Mice. Male NMRI mice (25 to 30 g) were purchased from Charles River (Sulzfeld, Germany). IFN-γR-deficient (IFN-γR−/−) mice (129/Sv) (11), LPS-nonresponder BALB/c mice carrying the mutated Tlr4 gene of C3H/HeJ mice (26), and the respective control mice were bred in the animal facilities of the Max-Planck-Institut für Immunobiologie (Freiburg, Germany). LBP-deficient (LBP−/−) mice and their heterozygous littermates (12) were bred in the Institut für Immunologie (Greifswald, Germany).

CLP. Mice were anesthetized by i.p. injection of 75 mg of Ketanest (Parke, Davis & Company, Munich, Germany)/kg of body weight and 16 mg of Rompun

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CLP and mortality was recorded (P < 0.12; log rank statistic).

Reagents. Recombinant mouse IL-12, kindly donated by M. Gatley (Hoffman LaRoche Inc., Nutley, N.J.), was injected i.p. as indicated above or after CLP. Recombinant mouse IFN-γ was kindly donated by G. R. Adolf (Bender GmbH, Vienna, Austria), and 1 μg was injected i.p. immediately after CLP. *P. acnes* serovar Minnesota 9700 was purchased from Difco Laboratories (Detroit, Mich.).

For neutralization of IFN-γ mice received 100 μg of protein A-purified rat anti-mouse IFN-γ monoclonal antibody R4-6A2 (24) i.p. immediately after CLP. To neutralize IL-12, mice received 1 ml of rabbit anti-mouse IL-12 antiserum (8) i.p. immediately after the operation. Control mice received 1 ml of normal rabbit serum.

Quantitation of tumor necrosis factor (TNF) serum titers and IFN-γ serum titers was done using the bioassays for mouse TNF (3) and IFN (16), respectively, as described earlier.

RESULTS

Role of LPS sensitivity in septic peritonitis. We compared the mortality of LPS-sensitive mice with that of LPS-resistant mice after CLP. BALB/c<1> mice (26) are highly resistant to LPS due to a point mutation in the intracellular domain of TLR4, which is critical for transducing LPS responses (22). The sensitivity of these mice to CLP as measured by the mortality was not different from that of mice with normal LPS responsiveness (Fig. 1).

Furthermore, ablation of the gene for LBP, a molecule which facilitates LPS responses (12), did not alter the mortality in the CLP procedure (Fig. 2).

Role of IL-12 in septic peritonitis. IL-12 has been shown on one hand to enhance mortality in LPS-induced shock (30) and on the other hand to be protective in a number of infection models (33). To determine whether endogenous IL-12 contributes to survival of mice after CLP-induced septic peritonitis, IL-12 was neutralized by treating mice with anti-IL-12 antiserum immediately after the operation. As can be seen in Fig. 3, there was no significant change in mortality due to this anti-IL-12 treatment, which has been shown in other experiments to effectively neutralize IL-12 in vivo (8).

Because exogenous IL-12 has been found to protect hosts from infections (19), we treated mice with recombinant IL-12 (rmIL-12). Administration of 10 to 100 ng of rmIL-12 immediately after CLP did not influence the outcome of CLP (Table 1).

### Table 1. Influence of rmIL-12 treatment on survival after sublethal CLP

<table>
<thead>
<tr>
<th>Time of rmIL-12 treatment</th>
<th>No. of mice surviving/no. tested after treatment with rmIL-12 at (ng per mouse):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Immediately after CLP</td>
<td>4/5</td>
</tr>
<tr>
<td>Day −1</td>
<td>5/5</td>
</tr>
<tr>
<td>Days −2 and −1</td>
<td>11/12</td>
</tr>
</tbody>
</table>

*Groups of mice were treated with the indicated amounts of rmIL-12 i.p. immediately after sublethal CLP (day 0) or on day −1 or both days −2 and −1. The data are given as cumulative survival of the animals over a period of 7 days after CLP. nt: not tested.*
TABLE 2. LPS-induced serum IFN-γ and TNF levels in untreated and IL-12 pretreated mice*

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>IFN-γ (U/ml) at (h):</th>
<th>TNF (U/ml) at (h):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>PBS</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>IL-12</td>
<td>120</td>
<td>480</td>
</tr>
</tbody>
</table>

* Groups of mice (n = 3) which had been pretreated on day −2 and −1 with phosphate-buffered saline (PBS) or 1 μg of rmIL-12 i.p. received LPS (10 μg) from S. enterica serovar Minnesota i.p., and IFN-γ and TNF levels were determined in serum prepared from blood taken 1 or 3 h after LPS challenge.

1). However, mice became more sensitive to CLP and the mortality increased when the animals were treated with 30 or 100 ng of rmIL-12 1 day prior to the operation or with 1 μg of rmIL-12 given two times before CLP.

In mice pretreated in this way twice with 1 μg of rmIL-12, a low nonlethal dose of bacterial LPS induced IFN-γ production. Compared to results for nonpretreated animals the IL-12 treatment led to serum IFN-γ titers that were more than 10-fold enhanced (Table 2) 3 h after LPS injection. The pretreatment with rmIL-12 also enhanced LPS-induced TNF serum levels very strongly. Thus, rmIL-12-pretreated mice exhibited enhanced sensitivity to LPS and were more sensitive to CLP.

**Role of IFN-γ in septic peritonitis.** To test whether IFN-γ is involved in the resistance to CLP-induced septic peritonitis, we compared the mortality of IFN-γR−/− mice and that of the respective wild-type (wt) mice in this model. As shown in Fig. 4b the IFN-γR−/− mice showed no change in mortality after CLP in comparison to control animals. Therefore we conclude that endogenous IFN-γ production is not essential for controlling the development of septic peritonitis after CLP. This was confirmed by finding that neutralization of endogenously produced IFN-γ after CLP did not affect the outcome of CLP (Fig. 5).

As shown above, pretreatment of mice with rmIL-12 enhanced mortality after CLP. IL-12 is a potent inducer of IFN-γ (1, 32). However, it is shown in Fig. 6 that pretreatment of IFN-γR−/− mice with rmIL-12 also enhanced lethality after CLP. This indicates that IFN-γ plays no role in the enhanced susceptibility to septic peritonitis induced by IL-12. However, administration of 1 μg of recombinant IFN-γ (rmIFN-γ) at the time of the operation significantly enhanced mortality in wild-type mice. This could be seen both after sublethal CLP (Fig. 7a) and after CLP which led to 40% lethality (Fig. 7b). The amount of IFN-γ injected was not lethal per se for uninfected mice (data not shown).

Bacterium-induced IL-12 production and the subsequent IFN-γ response are the essential events involved in the sensitization of mice by P. acnes to LPS (5, 6) and some (but not all) of the other biologically active components of gram-negative and gram-positive bacteria (18). In this study we compared the sensitivities of P. acnes-primed and unprimed wild-type and IFN-γ−/− mice to CLP. Surprisingly, no difference in susceptibility to CLP-caused death between control and sensitized mice and between wild-type and IFN-γR-deficient mice was
DISCUSSION

The protective function of IL-12 and IFN-γ in a number of infection models has been reported (1, 32, 33), and impaired IL-12 production was correlated with increased susceptibility to postoperative sepsis in patients (10). At the same time IL-12 and IFN-γ have been found to be deleterious in LPS-induced shock (5, 30) or after high-dose infection with gram-negative bacteria (Gumenscheimer and Freudenberg, unpublished data). We therefore attempted to clarify the role of IL-12 and IFN-γ in a clinically relevant model of sepsis. The response to CLP, a model of a postoperative mixed sepsis, is clearly independent of the activation of the innate immune system via LPS, the principal endotoxic molecule from the outer cell walls of gram-negative bacteria. Mice (BALB/c/J) in which the sensing of LPS, and thus of gram-negative bacteria, is impaired were as sensitive in this CLP model as LPS-competent mice. This finding is in full agreement with the report of Mercer-Jones et al. (17), who obtained the same results with C3H/HeJ mice, which have the identical genetic defect. Furthermore, the fact that the presence or absence of LBP did not alter the outcome after CLP also indicated that the capacity to sense LPS as a danger molecule during infection with gram-negative bacteria (9) does not seem crucial for survival in this model. Even though gram-negative bacteria constitute part of the infection after CLP (28), it seems clear that constituents other than LPS dominate the initiation of the innate immune responses essential for localization of the septic focus and the induction of effective antibacterial mechanisms (4). In agreement, a strong enhancement of LPS susceptibility in mice by P. acnes did in no way influence the experimental outcome after CLP in this study. This is remarkable, since pretreatment with P. acnes does not sensitize animals to LPS and, thus, to gram-negative bacteria only. As shown recently, the pretreatment of mice with P. acnes increased the susceptibility to a not yet identified component of gram-positive Listeria monocytogenes and to a macrophage-activating lipopeptide (MALP-2) of Mycoplasma spp. (18).

Keeping in mind that LPS is not the key molecule determining survival after CLP, the relevant mechanisms for survival remain to be determined. Obviously, these mechanisms are different from those induced by LPS. Clearly, IFN-γR activation is not critical for survival in CLP-induced septic peritonitis. This is in sharp contrast to the situation after infection with several other microbial organisms. It is also in contrast with the finding of the protective role of IFN-γ in a different septic peritonitis model, called CASP (31). The explanation for the different IFN-γ requirements in the two peritonitis models with mixed bacterial infection might have its roots in the critical need for abscess formation after CLP in order to localize the septic focus (4). In contrast to the largely localized inflammatory response after CLP, a systemic response after CASP, which more closely resembles that developing after challenge with LPS, may be involved in survival. The differences between

FIG. 6. Increased CLP susceptibility caused by IL-12 pretreatment did not depend on the IFN-γR. Groups of IFN-γR−/− mice (n = 10) and normal 129/Sv mice (n = 7) received either rmIL-12 (100 ng per mouse) or phosphate-buffered saline (PBS) 24 h before CLP i.p. Mortality was recorded (P < 0.0014 for IFN-γR−/− and IL-12 versus IFN-γR−/− and PBS, and P < 0.0009 for normal 129/Sv mice treated with IL-12 versus normal 129/Sv mice treated with PBS; log rank statistic).

FIG. 7. Postoperative administration of IFN-γ increased CLP lethality. (a) Immediately after sublethal CLP, groups of NMRI mice (n = 5) received either phosphate-buffered saline (PBS) or rmIFN-γ (1 μg per mouse i.p.). Mortality was recorded (P < 0.049; log rank statistic). (b) Immediately after CLP of low lethality, groups of NMRI mice (n = 5) received either PBS or rmIFN-γ (1 μg per mouse i.p.). Mortality was recorded (P < 0.004; log rank statistic).


