Role of Acute-Phase Proteins in Interleukin-1-Induced Nonspecific Resistance to Bacterial Infections in Mice

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Treatment with a single low dose (80 to 800 ng) of interleukin-1 (IL-1) 24 h before a lethal bacterial challenge of granulocytopenic and normal mice enhances nonspecific resistance. Since IL-1 induces secretion of acute-phase proteins, liver proteins that possess several detoxifying effects, we investigated the role of these proteins in the IL-1-induced protection. Inhibition of liver protein synthesis with Β-galactosamine (GALN) completely inhibited the IL-1-induced synthesis of acute-phase proteins. GALN pretreatment abolished the protective effect of IL-1 on survival completely (neutropenic mice infected with Pseudomonas aeruginosa) or partially (nonneutropenic mice infected with Klebsiella pneumoniae). Pretreatment with IL-6, a cytokine induced by IL-1, did not reproduce the protection offered after IL-1 pretreatment, nor did it enhance or deteriorate the IL-1-enhanced resistance to infection. A protective effect of IL-1 via effects on glucose homeostasis during the acute-phase response was investigated by comparing plasma glucose levels in IL-1-treated mice and control mice before and during infection. Although glucose levels in IL-1-pretreated mice were somewhat higher in the later stages of infection, no significant differences from levels in control mice were present, and the glucose levels in control-treated animals never fell to hypoglycemic values. We conclude that the IL-1-induced nonspecific resistance is mediated neither by the induction of IL-6 nor by the effects of IL-1 on glucose homeostasis. Acute-phase proteins generated after IL-1 pretreatment, however, seem to play a critical role in the IL-1-induced protection to infection.

Administration of the proinflammatory cytokine interleukin-1 (IL-1) has been shown to enhance nonspecific resistance in animals to several gram-positive and gram-negative bacteria, fungi, and plasmodia (35). The exact mechanism of this protection is still unclear. A direct antimicrobial effect of IL-1 in vitro (30) has been excluded, and enhanced clearance of microorganisms induced by IL-1 in vivo has not been a constant finding (22, 30). The protective effect of IL-1 to infectious challenges in granulocytopenic mice indicates that an effect on neutrophils does not mediate the IL-1-induced resistance (30, 35). Additional studies have provided evidence against a major role of IL-1-induced cyclooxygenase metabolites, glucocorticosteroids, or cytokines like IL-6 or tumor necrosis factor (TNF) in the IL-1-induced protection (30, 33–35).

In the present study, we investigated whether the effects of IL-1 on the acute-phase response could be responsible for the enhanced resistance induced by IL-1.

The acute-phase response comprises a constellation of metabolic, endocrinologic, neurologic, and immunologic alterations which starts within hours of an initiating stimulus. These changes appear to be meant to balance or curtail the potential adverse consequences of the inflammatory process and restore homeostasis (15). Synthesis of acute-phase proteins by the liver is a major component of the acute-phase response, and IL-1 appears to be an important inducer. We addressed the issue of whether enhanced resistance to infection after preexposure to IL-1 might be due to IL-1-induced enhanced secretion of acute-phase proteins using Β-galactosamine (GALN), a liver-specific blocker of RNA and protein synthesis which acts by depleting UTP and thereby is able to block protein synthesis in the acute phase of infection (5). We investigated the extent to which GALN inhibits the IL-1-induced acute-phase protein secretion by the liver. Next, we assessed whether GALN would also inhibit the IL-1-induced enhanced survival of normal and neutropenic mice after a lethal gram-negative bacterial challenge. Since IL-1 has been shown to induce IL-6 production by endothelial cells, fibroblasts, macrophages, and other cell types, it has been proposed that IL-6 is the direct mediator of a number of activities that were originally attributed to IL-1 (11, 32). IL-6 induces a series of acute-phase proteins and in addition possesses some other potentially protective effects during an infection (12, 32). Hence, we studied the role of IL-6 in IL-1-induced protection.

Another component of the acute-phase response to trauma and infection is carbohydrate dyshomeostasis. During severe infection, especially in rodents, an initial hyperglycemia may devolve into a profound hypoglycemia, the latter being a sign of decompensation (7). Since IL-1 affects glucose metabolism in various ways (29), we addressed the question of whether the effects of IL-1 on glucose homeostasis resulted in enhanced survival.

(Materials and methods)

Mice. Female outbred Swiss mice (weight, 20 to 25 g; TNO, Rijswijk, The Netherlands) were kept under specific-pathogen-free conditions with a light-dark cycle of 12 h (lights on at 7 a.m., lights off at 7 p.m.). Standard lab chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and

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Acidified water were available ad libitum. Granulocytopenic mice received irradiated food.

**Materials.** Recombinant human IL-1β (rhIL-1β) was kindly provided by P. Graber (Glaxo, Geneva, Switzerland) and P. Ghiara (Sclavo, Siena, Italy). According to the acidified water were available ad libitum. Granulocytopenic mice received irradiated food.

Bacteria for infectious challenges were obtained at 95°C for 30 min. To all these solutions, normal mouse serum was added to an end volume of 2% (vol/vol). GALN was purchased from Merck, Darmstadt, Germany. Glycated human IL-1β and IL-6 were kindly provided by P. Graber (Glaxo, Geneva, Switzerland) and Stutz, Sandoz Research Institute, Vienna, Austria. Endotoxin contamination was <0.4 U/mg in the Limulus assay.

**Experimental procedures.** All experiments started at 9:30 a.m. (time zero).

(i) Liver enzymes, acute-phase proteins, and IL-6 and glucose levels after treatment with IL-1 and/or GALN. For assessment of liver injury by GALN, aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels were determined in sera obtained from mice 24 h after intraperitoneal (i.p.) injection of different doses of GALN, 400 ng of rhIL-1β or PBS. Determinations of acute-phase proteins and IL-6 levels, mice received two injections. GALN or vehicle (saline) was given i.p. at time zero, and IL-1 control (heat-inactivated IL-1) was administered i.p. (0.1 ml) at time zero. At various time points, blood was taken retro-orbitally from ether-anesthetized mice to prepare EDTA-treated plasma (fibrinogen measurements) or serum (serum amyloid A [SAA] and IL-6 measurements). IL-6 levels were also measured in serum obtained at different time points after a single i.p. treatment with 800 ng of rhIL-1β or 800 mg of rhIL-6.

In the experiments in which glucose was measured, mice received a single i.p. injection of 800 mg of IL-1 or vehicle at time zero, an intramuscular (i.m.) challenge with K. pneumoniae at 24 h, and gentamicin (120 mg/kg subcutaneously) 6 h later. Trunk blood of decapitated mice was collected at several time points in fluoride-coated tubes on melting ice. All plasma and serum samples were stored at −20°C until assay.

When neutropenic mice were used, neutropenia (<0.5 × 10⁹ granulocytes per liter) was induced by two subcutaneous injections of cyclophosphamide (150 and 100 mg/kg, respectively) on days −3 and 0.

(ii) Effects of cytokine and/or GALN pretreatment on survival. For survival experiments, some mice were rendered neutropenic as described above. IL-1 or control treatment (heat-inactivated IL-1) was administered i.p. (0.1 ml) at time zero. GALN (18 mg per mouse) or vehicle (saline) was given i.p. 30 min earlier. The infectious challenge was administered at 24 h. Neutropenic mice received P. aeruginosa i.m.

(0.5 × 10⁸ to 1 × 10⁹ CFU per mouse). Because P. aeruginosa does not kill nonneutropenic mice unless very high inocula are used, such mice received K. pneumoniae (1 × 10⁶ to 5 × 10⁶ CFU per mouse). Six hours after the infectious challenge, 120 mg of gentamicin per kg of body weight was given subcutaneously in order to postpone the time of death and thus accentuate the differences between treatment groups. Mortality was recorded for a period of at least 48 h after challenge.

In investigations on the protective effect of human and murine IL-6 on P. aeruginosa infection in neutropenic mice, the experimental protocol described above was used. The cytokines were given i.p. (0.1 ml) at time zero, and when both IL-1 and IL-6 were administered, a mixture of both cytokines was prepared immediately before injection. In all cases, heat-inactivated IL-1β was given as the control treatment.

**Assays.** Serum ASAT and ALAT levels were assessed by routine procedures (BM/Hitachi 747; Boehringer Mannheim, Germany). Plasma glucose concentrations were determined by the hexokinase method (Cobas Bio; Hoffmann-LaRoche B.V., Mijdrecht, The Netherlands). IL-6 levels in serum were quantitated as hybridoma growth factor activity by using the IL-6-dependent murine hybridoma 7TD1 (a kind gift from J. van Snick) as described previously (28). Hybridoma growth factor activity resulting in half-maximal stimulation of the target cell growth was arbitrarily defined as 1 U. The reference standard in these experiments was rhIL-6 (specific activity, 10⁷ U/ml; a kind gift from Immunex Corp., Seattle, Wash.). The detection limit of the assay was 50 U/ml. SAA was measured by a modification of the direct-binding enzyme-linked immunosorbent assay method for human plasma (27) by using a mixture of rabbit polyclonal antiserum to mouse SAA1 and SAA2 (kind gift from J. Sipe, Boston, Mass.). Assays for fibrinogen in EDTA-plasma were performed by measuring the rate of conversion of fibrinogen to fibrin in the presence of thrombin exces. Measurements of fibrin clot formation were performed on a couagulometer (KC-10; Salm & Kipp, Breukelen, The Netherlands). The results are expressed as milligrams of fibrinogen per liter of plasma; the sensitivity of the assay was 1,200 mg/liter. Endotoxin levels were assessed by the Limulus amoebocyte lysate gelation test, which had a sensitivity of 6 pg/ml.

**Statistical analysis.** Survival curves were analyzed by the log rank test (24). Comparisons of medians of liver enzymes, acute-phase proteins, IL-6 levels, and glucose levels between treatment groups were made by the Kruskal-Wallis test at each time point (13). When five components were analyzed, the Bonferroni-corrected α = 0.05/5 = 0.01 was used.

**RESULTS**

**Hepatotoxicity of GALN.** Since GALN is potentially hepatotoxic (5), we first assessed the effect in Swiss mice of different doses of this hexosamine on ASAT and ALAT levels in serum at 24 h after i.p. injection. At this time point, liver damage should have developed (5). After administration of 18 and 36 mg of GALN per mouse (720 and 1,440 mg/kg, respectively), doses which are commonly used by others (1, 18, 37) and which are reported to be effective in the inhibition of protein synthesis (1, 5), serum ASAT and ALAT levels in normal mice did not differ significantly from those in vehicle-treated mice; 90 and 180 mg of GALN per mouse induced a significant increase in the levels of both enzymes compared with the levels in vehicle-treated mice.
IL-1-induced resistance to infection

Before the IL-1 injection significantly inhibited the plasma fibrinogen levels, 3,000 mg/liter after IL-1 treatment and fibrinogen at 24 h.

Plasma fibrinogen levels in GALN-treated normal mice 24 h after i.p. injection of PBS, 400 ng of IL-1, or different doses of GALN (six to eight mice per group). In comparison with PBS-treated mice, enzyme levels were significantly elevated after administration of 90 mg of GALN (ASAT, \( P = 0.007 \); ALAT, \( P = 0.0027 \)) and 180 mg of GALN (\( P = 0.009 \) for both ASAT and ALAT). Bars indicate the results for mice treated as follows (from left to right, respectively): PBS, 400 ng of IL-1, 18 mg of GALN, 36 mg of GALN, 90 mg of GALN, and 180 mg of GALN.

Effects of pretreatment with IL-1 and/or GALN on survival. Pretreatment of nonneutropenic mice with 800 ng of IL-1 24 h before an i.m. infection with \( K. \) pneumoniae significantly enhanced survival (\( \chi^2 = 25.93; \ P < 0.0005 \)) (Fig. 3A). Administration of GALN 30 min before the IL-1 pretreat-
ment partially inhibited the IL-1-induced protection, with significant differences in this group compared with the levels of protection in both the IL-1-treated group and the group treated with GALN alone. GALN alone did not differ significantly from survival of mice treated with vehicle (χ² = 0.22). In neutropenic mice infected with *P. aeruginosa*, IL-1 pretreatment significantly enhanced survival (χ² = 13.10; P < 0.0005) (Fig. 3B). GALN injection 30 min before the IL-1 injection completely inhibited the protective effect of IL-1, with no difference in survival between this group and mice treated with GALN and then vehicle or with GALN alone (χ² = 0.00). Mice treated with GALN survived somewhat better than mice treated with vehicle, but this difference was not significant. All normal and neutropenic mice treated with GALN without subsequent infection survived.

Role of IL-6 in IL-1-induced protection to infection. Serum IL-6 levels measured 2 h after administration of 800 ng of IL-1 were not reduced by pretreatment with GALN given i.p. (18 mg per mouse) in normal (Fig. 2C) and neutropenic mice. On the contrary, IL-6 levels after GALN plus IL-1 treatment were enhanced in comparison with those in mice treated with IL-1, an enhancement which did not reach significance in normal mice (P = 0.026) and neutropenic mice (P = 0.34) (median levels, 58,300 and 47,350 U/ml, respectively; seven mice per group). GALN administration alone does not elicit an IL-6 response. In further investigation of a putative role of IL-6 as a mediator of the IL-1-induced protection to infection, serum IL-6 concentrations were measured. IL-6 levels peaked at 30 min after an i.p. injection of 800 ng of rhIL-6 (38,900 U/ml) and at 2 h after i.p. injection of 800 ng of IL-1 (5,020 U/ml) (six mice per datum point). However, no protective effect of pretreatment with 80, 400, or 800 ng of IL-6 was observed (Fig. 4A). To investigate whether some degree of species specificity of IL-6 (17, 21) plays a role, we also assessed the effect of mIL-6 (80, 400, 800, and 1600 ng) on survival of *P. aeruginosa* infection in neutropenic mice, but this molecule also did not influence survival (Fig. 4B).

Since several studies report synergistic effects between IL-1 and IL-6 (4, 11), we assessed whether IL-6 would enhance IL-1-induced protection to a lethal *P. aeruginosa* infection. However, administration of 80 or 800 ng of hIL-6 or mIL-6 did not affect protection induced by 80 or 800 ng of IL-1 (shown for mIL-6 in Fig. 4C).

The deleterious effects of IL-6 in infections have also been suggested in several reports (31, 36). We addressed this issue by administering higher doses of IL-6 prior to *P. aeruginosa* infection, but we observed that survival of mice pretreated with rhIL-6 doses of 1.6, 4, and 8 μg and 1.6 μg of mIL-6 per mouse did not differ significantly from survival of control-treated mice (Fig. 4D). Because of the complete absence of an effect of IL-6, we did not perform similar studies in nonneutropenic mice.

**Effect of IL-1 treatment on plasma glucose concentration before and after infection.** Experiments examining the effect of IL-1 treatment on the plasma glucose concentration before and after infection were limited to mice infected with *K. pneumoniae*, because fewer pretreatments, which could possibly influence glucose metabolism, are necessary in this model. The plasma glucose concentrations (median values) in mice after an i.p. injection of 800 ng of IL-1 at time −24 h and *K. pneumoniae* infection by i.m. injection of *K. pneumoniae* at time zero are depicted in Fig. 5. The plasma glucose concentration showed a nadir at 4 h after i.p. injection in IL-1-treated mice but not in vehicle-treated mice (4.85 and 8.5 mmol/liter, respectively). The difference between IL-1-treated and vehicle-treated mice was significant at 4 and 12 h after IL-1 injection; and also at 24 h after IL-1 injection, the time point of infectious challenge, the glucose level was significantly lower than that in control mice. The glucose levels in IL-1-treated mice returned to control levels at 36 h after IL-1 injection.

Median plasma glucose levels after i.m. infection with *K. pneumoniae* did not differ significantly between the treatment groups, with the exception of the level at 2 h, when values in the IL-1-treated group were still lower, and with the level at 48 h after infection, when values in the IL-1-treated group were higher. In late stages of the infection, there is a tendency for mice pretreated with IL-1 to have higher glucose levels than those in mice given the control treatment. At this stage, signs of discomfort in the infected animals (piloerection, decreased physical activity) were also less pronounced in the IL-1-treated group. The IL-1-pretreated mice all survived for 48 h, whereas approximately 20% of the control-treated mice died during this period.

**DISCUSSION**

We have previously reported that a single low dose of IL-1 administered 24 h, but not 6 or 0.5 h, before a lethal...
gram-negative bacterial infection enhances survival (30, 32a). In the present study, we demonstrated that this enhanced survival is associated with an increased level of production of acute-phase proteins. Acute-phase proteins, which are synthesized by the liver during a variety of acute inflammatory processes (15), may operate as oxygen radical scavengers, protease inhibitors, coagulation factors, and opsonins, and may therefore play a role in the nonspecific defense of the host to infections. Most acute-phase proteins are induced by IL-1 within a time course similar to that required for induction of protection in a lethal infection. To investigate the role of acute-phase proteins in the IL-1-induced enhanced resistance, we used GALN, a hepatocyte-specific inhibitor of RNA and protein synthesis (5). In normal and neutropenic mice, the IL-1-induced synthesis of acute-phase proteins was completely blocked by GALN pretreatment. The protective effect of IL-1 pretreatment on infection was inhibited partially (nonneutropenic mice infected with *K. pneumoniae*) or completely (neutropenic mice infected with *P. aeruginosa*) by a GALN dose that did not damage liver parenchymal cells. GALN did not inhibit production of IL-1-induced IL-6, confirming the expected specificity of the effects of GALN on hepatocytes (which do not produce IL-6), not affecting protein synthesis in IL-6-producing cells. IL-6 concentrations in mice treated with GALN and IL-1 were even somewhat enhanced in comparison with those in mice treated with only IL-1 (2.7-fold increase in normal mice; 1.2-fold increase in neutropenic mice). GALN per se did not increase IL-6 levels, arguing against an effect of traces of contaminating endotoxin (less than 0.7 pg per mouse). GALN itself may have a slight priming effect on macrophage cytokine production, as observed by some investigators in vitro (16) and in vivo (18) after an endotoxin trigger. Others did not find such an effect (1). We do not believe that a GALN-induced sensitizing effect of macrophages for IL-1, resulting in increased mac-
Uridine supplementation (1, 16) showed an inhibition by GALN of the protective effect of IL-1 (18). In addition, the sensitizing effect of GALN on endotoxin or TNF toxicity was shown to be mediated by inhibition of hepatic metabolism and not by activation of macrophages, since this sensitization can be completely reversed by restoring hepatic RNA and protein synthesis with uridine supplementation (1, 8, 19).

The lack of an effect of GALN pretreatment on survival of infection per se also argues against a detrimental effect of GALN via mechanisms independent of IL-1-induced protection, e.g., by an aspecific toxic effect of GALN or by GALN-induced sensitization for bacterial products or cytokines like TNF (8, 37), and against a deleterious effect of the reduced serum fibrinogen levels observed in GALN-treated mice. Taken together, these results suggest a critical role of liver-induced proteins, most conceivably acute-phase proteins, in the IL-1-induced nonspecific resistance to infection.

A role of acute-phase proteins in IL-1-induced enhanced survival of several challenges has also been suggested by other investigators. GALN abolished IL-1-induced protection to a subsequent lethal dose of IL-1 or TNF (37), and GALN inhibited enhanced survival of a lethal dose of lipopolysaccharide or TNF after a turpentine-induced acute-phase response in mice (1). Libert and coworkers (19) showed an inhibition by GALN of the protective effect of IL-1 pretreatment to a lethal challenge of TNF-α and GALN, an inhibition which could be restored by the addition of uridine. Those authors also suggested a role of acute-phase proteins in the IL-1-induced protection (19).

IL-1 induces IL-6, and several effects of IL-1 have been reported to be mediated by IL-6 (11, 32). Apart from induction of acute-phase proteins, IL-6 has other potentially beneficial effects (6, 14, 26, 32). Nevertheless, our observations plead against a role of IL-6 in the IL-1-induced enhanced resistance to infection. First, although GALN inhibits IL-1-induced protection, it does not inhibit IL-1-induced IL-6 production. Second, doses of 80 to 800 ng of human IL-6 did not enhance survival after a lethal P. aeruginosa challenge, despite the larger IL-6 peak in mice after rhIL-6 treatment than that after IL-1 treatment. Higher doses (1.6 to 8 μg) neither protected nor showed deleterious effects, in accordance with earlier reports by us and others (4, 32). Since there is some species specificity for IL-6 (17, 21), we tested the protective effect of pretreatment with different doses of mIL-6, but no effect on survival was observed. Some studies describe a synergistic effect between IL-1 and IL-6 (4, 11), and IL-1 and IL-6 are likely to be present simultaneously after IL-1 injection. However, we found that coadministration of different doses of rhIL-6 or mIL-6 did not enhance the IL-1-induced enhanced survival of infection.

Other investigators reported protection by IL-6 pretreatment against a lethal combination of lipopolysaccharide and GALN, provided that the induced TNF was partially neutralized (2). Using very high doses of mIL-6 (20 to 50 μg), another group found enhanced clearance of Listeria monocytogenes (20). However, in accordance with our results, pretreatment with 0.2 to 5 μg of mIL-6 did not protect against a lethal combination of recombinant murine TNF and GALN, whereas IL-1 did, according to the authors (19), by induction of acute-phase proteins. In addition, our results showing high IL-6 levels associated with the lack of protection to infection in mice pretreated with GALN and IL-1 are similar to the high serum IL-6 levels reported by Havell and Sehgal (10) in anti-TNF-α-treated mice that succumbed to experimental listeriosis. The fact that IL-6 as an inducer of acute-phase proteins is not capable of enhancing nonspecific resistance, whereas acute-phase proteins themselves seem to do so, is not necessarily contradictory. IL-6 and IL-1 are known to induce different spectra of acute-phase proteins (25), and it is conceivable that the IL-1-induced spectrum is protective and the IL-6-induced spectrum is not. Furthermore, IL-1 is a more potent inducer than IL-6 for several proteins that can be induced by both IL-1 and IL-6 (21, 23, 25).

We also investigated the role of IL-1-induced changes in blood glucose levels. IL-1 affects glucose homeostasis in different ways (7, 29). Whereas insulin-inducing and insulin-like effects would lead to hypoglycemia, the toxic effects of higher doses of IL-1 on pancreatic β cells and the induction by IL-1 of glucagon and stress hormones would elevate plasma glucose levels. These effects may result in a reduction of hypoglycemia during the terminal phase of sepsis. Although the fall in blood glucose levels induced by IL-1 was not completely restored at the time of infection in our mice, the plasma glucose levels measured in infected mice after IL-1 treatment did not differ significantly from those in controls at most time points. Moreover, in both treatment groups, glucose levels never fell to hypoglycemic values. Therefore, effects on blood glucose levels do not seem to play a major role.

In conclusion, the data presented here suggest that the increase in resistance induced by IL-1 is at least partially mediated by GALN-inhibitable liver proteins, most likely acute-phase proteins. IL-6 does not seem to be an intermediate for this IL-1 effect. Finally, the improved outcome of infection in IL-1-treated mice is not associated with physiologically significant changes in blood glucose levels during infection.

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