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A low dose of recombinant interleukin 1 protects granulocytopenic mice from lethal Gram-negative infection

(immunomodulation/natural resistance/macrophages)

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ABSTRACT Natural and synthetic immunomodulators that increase nonspecific resistance to infection induce interleukin 1 (IL-1) production. Therefore, we investigated the effect of the administration of IL-1 on the survival of lethally infected granulocytopenic mice. Mice with cyclophosphamide-induced granulocytopenia were injected with approximately 10⁷ Pseudomonas aeruginosa in the thigh muscle at time 0; gentamicin was administered 6 hr and 23 hr later. When recombinant human IL-1β (one of the two forms of IL-1) was given as a single i.p. injection 24 hr before the infection, survival was increased. Using 80 ng of IL-1β per mouse, survival compared to control animals was 98% vs. 71% at 24 hr, 98% vs. 60% at 30 hr, 86% vs. 36% at 36 hr, and 61% vs. 11% at 48 hr (P < 0.001) after the infection. No effect of IL-1β was observed when it was given 0.5 hr before or 6 hr after the infection. Animals not treated with gentamicin also benefited from the IL-1β. Administration of the cyclooxygenase inhibitor ibuprofen did not affect the activity of IL-1β. Numbers of bacteria cultured from the blood, thigh muscle, liver, spleen, and kidney were similar in IL-1-treated and control animals. Superoxide production by peritoneal macrophages was also similar in the two groups. These studies demonstrate that IL-1 pretreatment protects granulocytopenic mice against lethal pseudomonas infection and suggest that this protection occurs through a noncellular mechanism.

For a number of decades, investigators have sought methods to enhance nonspecific resistance to infection (1–4). Several substances have been found that are able to increase natural resistance mechanisms. Most of these substances, such as lipopolysaccharides (2), BCG (bacillus Calmette–Guérin), and muramyl peptides (4) are derived from bacteria, and their administration has led to considerable toxicity. Despite efforts to modify toxicity (e.g., by the preparation of analogues (4)), these immunomodulatory substances have not gained acceptance as a therapy in humans.

The increase in natural resistance to infection produced by these substances may be due to enhancement of humoral (5) and/or cellular defense mechanisms. With respect to humoral defense, hepatic acute-phase proteins and other nonspecific factors may play a role; with respect to cellular defenses, stimulation of mononuclear phagocytes is considered a major mechanism (6).

Substances that increase nonspecific resistance appear to be able to stimulate mononuclear phagocytes to synthesize and secrete interleukin 1 (IL-1) in vitro (7). IL-1 is the collective name for two 17-kDa proteins (IL-1α and IL-1β) that have a number of important biological effects (7, 8). Because IL-1 is a major mediator of fever and other acute-phase responses, it is possible that many of the effects of the immunomodulatory substances mentioned above are mediated by this molecule. Therefore, we investigated the ability of recombinant IL-1 to ameliorate the outcome of a lethal bacterial infection in mice. We chose to study granulocytopenic mice in order to simulate the situation in neutropenic patients, in whom Gram-negative bacteremia is relatively common and the prognosis is grave. A portion of this work has previously been presented.

MATERIALS AND METHODS

Mice. Female, 25-g Swiss–Webster mice (Taconic Farms, Germantown, NY) were kept in cages (six mice per cage) with filter lids and were fed standard lab chow and water ad libitum.

IL-1. Recombinant human IL-1β (kindly supplied by Cis­tron Biotechnology, Pine Brook, NJ) that contained less than 60 pg of endotoxin per mg of protein was used in the majority of the experiments. Recombinant IL-1β from a different source (kindly provided by Alan Shaw, Biogen, Geneva) was also used in some experiments.

The IL-1 was given as a single i.p. injection in 2% (vol/vol) normal mouse serum in 0.1 ml of pyrogen-free saline. Control mice received heat-inactivated (100°C for 20 min) IL-1. For the experiments with peritoneal macrophages (see below), IL-1 was given i.m.

Infection Models. Mice were rendered granulocytopenic (<0.5 × 10⁹ granulocytes per liter) by means of i.p. injec­tions of cyclophosphamide (Bristol Myers) at 150 and 100 mg/kg of body weight 4 days and 1 day, respectively, before the inoculation of 1–2 × 10⁷ Pseudomonas aeruginosa (ATCC no. 27853) in the thigh muscle as described elsewhere (9, 10). Unless stated otherwise, doses of gentamicin (Lyomed, Rosemont, IL) at 120 mg/kg were given s.c. 6 and 23 hr postinfection.

A similar infection was established using Streptococcus pneumoniae [10⁶ colony-forming units (cfu) per mouse] obtained from the cerebrospinal fluid of a patient with pneumococcal meningitis. The time schedule for these experiments was the same as with the P. aeruginosa. No antibiotic treatment was given except in a few experiments, in which benzylpenicillin (Pfizer) at 12 mg/kg was adminis­tered s.c. 6 and 23 hr after infection.

The mice in each cage were randomized to receive either IL-1 at different time points and in different dosages or heat-inactivated IL-1. Survival was scored over a period of at least 48 hr. In one series of experiments with the pseudomonas model, mice were pretreated with a single i.p. injection of the cyclooxygenase inhibitor ibuprofen (Upjohn) at 10 mg/kg. For the experiments involving peritoneal

Abbreviations: IL-1, interleukin 1; cfu, colony-forming units.

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macrophages (see below), the two dosages of cyclophosphamide were administered s.c.  

Peripheral Blood Leukocytes and Peritoneal Macrophages.  

Blood leukocytes obtained by cardiac puncture were counted in a hemocytometer, and smears were stained with Diffquick (AHS Del Caribe, Aguada, Puerto Rico) for leukocyte identification. Peritoneal cells were harvested as described elsewhere (11), washed twice in Ca2+/Mg2+-free Hank's balanced salt solution, and counted. Preparations for light microscopy were made using a cytocentrifuge (Shannon Instruments) and Diffquick stain.

The production of superoxide by peritoneal macrophages, as measured by the superoxide dismutase-inhibitable reduction of cytochrome c (Sigma) with 100 µM phorbol 12-myristate 13-acetate (Sigma) as a stimulus, was assayed as described elsewhere (12).

In Vitro Antibacterial Effects of IL-1. We examined the possibility that IL-1 might have a direct antibacterial effect or might potentiate the antibacterial effect of gentamicin against _P. aeruginosa_. Using an automated spiral plater (Spiral Systems, Cincinnati, OH), we prepared a concentration gradient of IL-1 ranging from 0.15 to 42 ng/ml on the agar surface of Mueller–Hinton plates. After the plates had been dried, some were subjected to a second spiral plating of gentamicin with a concentration gradient ranging from 0.2 to 30 µg/ml. Radial streaks of _P. aeruginosa_ from a suspension containing 10^5 cfu/ml were made on the plates. After incubation overnight at 37°C, the distance from the center to the most central point of growth inhibition was measured. The effect of IL-1 alone was determined by comparing zones of inhibition in plates with and without IL-1. The effect of IL-1 on the activity of gentamicin was determined by comparing the minimal inhibitory concentration of gentamicin in plates with and without IL-1.

Clearance of Bacteria. Twenty-four hours after pseudomonas infection, the granulocytopenic animals were killed by carbon dioxide asphyxia. Immediately after death, blood cultures were taken by cardiac puncture, and the muscles of the left thigh (the site of inoculation of the bacteria), the spleen, the kidney, and the liver were removed aseptically. The tissue samples were weighed and were homogenized in sterile saline in a tissue grinder. To bring the number of cfu into the optimal range for reading, samples of thigh muscle were diluted 1:10,000 and other samples were diluted 1:10 in sterile saline. The suspensions were then plated on sterile Mueller–Hinton agar using the spiral plater. After overnight incubation at 37°C, the number of cfu was counted.

Statistical Analysis. Survival curves were analyzed using the Kaplan–Meier logarithm of rank test (13). Student’s t test was used to analyze the results of granulocyte numbers and macrophage function studies.

RESULTS

Survival of Mice. Human recombinant IL-1/β, given as a single i.p. injection of 80 ng, did not influence the survival of infected mice when it was given either shortly before or 6 hr after infection of _P. aeruginosa_. However, when IL-1 was given 24 hr before infection, dramatic improvement of survival was observed compared to the survival of controls ($\chi^2 = 26.5, P < 0.001$) (Fig. 1A). The effect of IL-1 on survival was dose-dependent; dosages of 800, 80, or 8 ng per mouse afforded protection, whereas 0.8 ng was without effect (Fig. 1B). In subsequent experiments we used the relatively low dose of 80 ng per mouse (3.0 µg/kg). The results obtained with recombinant IL-1/β from the two sources were similar.

When gentamicin treatment was withheld, the differences in survival between IL-1-treated mice and controls were still apparent (Fig. 1C). Pretreatment with a single injection of ibuprofen, given s.c. 30 min before the injection of IL-1, did not significantly influence the survival of either the IL-1-treated group or the control group.

In mice infected with _S. pneumoniae_, the differences between IL-1-treated and untreated mice were less prominent. Although we observed increased survival with IL-1 in some experiments, the overall reproducibility of the IL-1 effect was less clear with this microorganism than with _P. aeruginosa_ (Fig. 2). Treatment with benzylpenicillin did not increase the differences between mice treated with IL-1 and controls.

Blood Leukocytes and Peritoneal Macrophages. At the time of infection and during the subsequent days, animals were profoundly granulocytopenic (Table 1). The recovery of granulocyte counts was approximately synchronous in mice treated with IL-1 and controls treated with heat-inactivated IL-1.

The number of macrophages obtained from the unperfused peritoneal cavities (cyclophosphamide given s.c. and IL-1 given i.m. for these experiments) was not different for IL-1-treated mice and controls (Table 1). Fewer than 0.5% of the peritoneal cells were granulocytes in these mice. Super-

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**Fig. 1.** Effect of IL-1β treatment on the survival of granulocytopenic mice. Recombinant human IL-1β was given as a single i.p. injection. Control mice received heat-inactivated (100°C for 20 min) IL-1. (A) Survival with IL-1 administered at different time points is depicted. There were 28–58 mice in each group. The survival with IL-1 given at −24 hr is significantly different from that in the control group ($P < 0.001$, $\chi^2 = 26.5$), whereas survival with IL-1 given at −30 min or +6 hr is not different from that in the control group. (B) Each group represents at least 21 mice. The survival rates are not significantly different between groups given 800 ng and 80 ng of IL-1, whereas survival rates in groups given 800 ng, 80 ng, or 8 ng are significantly different ($P < 0.05$) from the survival rates in the control animals and those given 0.8 ng. (C) The number of mice is at least 18 per group. IL-1 was given at −24 hr at a dose of 80 ng. The difference in survival between IL-1-treated mice not treated with gentamicin and mice that received no treatment is significant at $P < 0.05$ ($\chi^2 = 6.26$).
oxide production by macrophages from mice of the two groups was measured in three experiments using pooled peritoneal macrophages from six mice per group. The differences between cells of mice treated with IL-1 and controls were not statistically significantly different (two-tailed t test).

In Vitro Assessment of Antimicrobial Activity. No direct antibacterial effect of IL-1 could be demonstrated when the pseudomonas organisms were incubated with concentrations of IL-1β up to 42 ng/ml. Also, IL-1 did not potentiate the effect of gentamicin on the bacterium. The minimal inhibitory concentration of gentamicin for *P. aeruginosa* was 1.8 mg/liter.

Clearance of *P. aeruginosa*. In two sets of experiments, no differences in the numbers of bacteria in blood and tissues were found between mice treated with IL-1 or with heat-inactivated IL-1 (Fig. 3). When the data were expressed as number of microorganisms per organ rather than per gram of tissue, the data from two groups still did not differ.

**DISCUSSION**

In these studies, we report the efficacy of a relatively low dose of IL-1β (3.0 and 0.3 μg/kg) in preventing death in severely leukopenic animals with a *P. aeruginosa* infection. These results hold promise that such treatment might decrease Gram-negative infections in patients with severe granulocytopenia. Patients whose granulocyte counts fall below 0.1 × 10^9 per liter are at high risk of acquiring Gram-negative bacterial infection (14, 15). In this circumstance, periodic prophylactic administration of IL-1, started when the number of granulocytes falls below 0.5 × 10^9 per liter, is a feasible option that should be explored further. It is likely that an equivalent dose of IL-1β employed in these studies can be used with acceptable toxicity in humans.

Because IL-1 is a major endogenous pyrogen (7, 8), an important side effect that can be anticipated is fever. Therefore, our finding that pretreatment with the antipyretic drug ibuprofen did not influence the protective effect of IL-1 is relevant. Other studies have shown that indomethacin, another cyclooxygenase inhibitor, in fact, increased nonspecific resistance to infection (16)

The results of our studies demonstrating a protective effect expand upon those obtained by Kampschmidt and Pulliam (17), who used a partially purified rat cytokine preparation in *Salmonella typhimurium* infection, and those reported recently by Ozaki et al. (18). The latter investigators used human recombinant IL-1α in nonneutropenic mice that were infected with either *P. aeruginosa* or *Klebsiella pneumoniae*.

In the present studies, the time of administration of IL-1 in relation to time of infection was critical to the outcome. These findings are reminiscent of those in earlier studies (1–3, 5) in which bacterial endotoxin was given to mice 1 hr prior to infection (i.e., "negative phase"). The lack of any beneficial effect when IL-1 was given shortly before infection with *P. aeruginosa* may also be explained by the rapidity of the spread of the bacteria in granulocytopenic mice. The observation (18) that IL-1 was effective when given at the time of infection with *Klebsiella* in nongranulocytopenic mice, in which host defenses may be adequate, is in agreement with this interpretation. A similar observation has been made previously with muramyl dipeptide in a model of intramuscular infection in normal mice (4).

The mechanisms responsible for the IL-1-induced increase in survival remain unclear. A direct antibacterial effect of IL-1 or a potentiation by IL-1 of the antibacterial effect of gentamicin was ruled out in *vitro*. The rapid clearance of i.p. injected recombinant IL-1β from the circulation (19) also makes a direct effect of the molecule on bacterial growth unlikely. Because the animals were profoundly granulocytopenic, an effect of IL-1 on the neutrophils was considered unlikely. Administration of IL-1 induces hypoferremia, which has an antimicrobial effect (20); however, this hypo-
The beneficial effect of IL-1 on hematopoietic recovery (22) were not demonstrated in our short-term experiments.

An effect of IL-1 on macrophages could explain many of our results. The number of peritoneal macrophages and the ability of these cells to produce superoxide were not influenced by treatment with IL-1. In this respect, the effects of IL-1 treatment do not completely mimic those of muramyl dipeptide on macrophages (12). The most convincing argument against an effect on macrophage activation comes from the bacterial clearance data, which failed to demonstrate a difference between IL-1-treated and control mice. Apparently, IL-1-treated mice survive better than control mice despite similar numbers of bacteria in their blood and in organs, including organs that contain the largest populations of mononuclear phagocytes.

The most likely explanation for the beneficial influence of IL-1 in our studies is through a humoral effect [for example, on acute-phase proteins such as an endotoxin-binding protein (23, 24)]. The time required for an optimal effect of IL-1 in our model fits well with the time needed for the synthesis of most acute-phase proteins. The death of control mice, which began to occur approximately 1 hr after the second dose of gentamicin, could in fact be due to liberation of endotoxin by this antibiotic (25). Another possibility is that the protective effect of IL-1 may involve production of substances that counteract the toxicity of pseudomonas exotoxins. Our results with pneumococcal infection were inconclusive and do not shed further light on the precise mechanism of protection induced by IL-1.

Two other favorable effects of IL-1 treatment in experimental animals [namely, protection from lethal radiation (26) and protection against oxygen damage (27)] are also presently unexplained, but, similar to our results, require 24-48 hr of pretreatment with IL-1.

Recent reports indicate that high dosages (>1.8 mg/kg) of recombinant interleukin 2 (IL-2) are also able to provide protection against death from infection in mice (28, 29). Because high concentrations of IL-2 induce macrophages to produce and secrete IL-1 (30), the beneficial effects of IL-2 can at least partially be explained by induction of IL-1.

In conclusion, prophylactic administration of a low dose of IL-16 in order to increase natural resistance to infection is potentially feasible in high-risk patients (e.g., those with declining granulocyte counts and at high risk for development of a Gram-negative infection). The synergism of IL-1 (which is identical to hematopoietin 1 (22]) with factors such as granulocyte-macrophage colony-stimulating factor on hematopoietic recovery could further contribute to the potential utility of IL-1 as a therapeutic agent, especially in granulocytopenic patients.

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