Amelioration by Muramyl Dipeptide of the Effect of Induced Hyperferremia upon *Klebsiella* Infection in Mice

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Received 12 April 1982/Accepted 29 June 1982

Induced hyperferremia enhanced local *Klebsiella* infection, with and without a surgical suture as a test foreign body. Both bacterial proliferation and death occurred often in mice treated with ferric ammonium citrate. Muramyl dipeptide significantly protected animals from local bacterial growth, death, and, to some extent, bacteremia.

Vaccination, asepsis, and antimicrobial agents have apparently altered the mortality and morbidity of some bacterial infections; however, in an era of radiation treatment, vigorous chemotherapy for neoplastic disease, and antibiotic-resistant microbes, these methods alone are often inadequate. Nonspecific enhancement of normal host defense mechanisms is another approach to the control of infection.

Freund established that delayed hypersensitivity and antibody production against various antigens can be enhanced by the administration of a water-in-oil emulsion of killed mycobacteria; however, that method of administration also causes adjuvant arthritis, autoimmune disease, and local granulomata (2). Other agents, such as *Mycobacterium bovis* BCG and *Propionibacterium acnes*, have also been examined for nonspecific enhancement of host defenses, but these agents cause problems of variable efficacy, inconsistent bioassays, occasional immunosuppression, and a very narrow therapeutic ratio (14, 17).

Some of these undesirable effects are thought to be caused by the incorporation of antigens other than those required for host defense enhancement. A dipeptide component of the *Mycobacterium* cell wall has been identified as the minimum component capable of enhancing the immune response. It has been synthesized as N-acetylmuramyl-L-alanine-D-isoglutamine, also known as muramyl dipeptide (MDP). It enhances the host response to infection when administered in an aqueous medium either orally or parenterally and is relatively free of side effects (2, 3, 9, 11).

MDP has been shown to have the following biological activities: enhancement of antibody production, enhancement of delayed-type hypersensitivity to tuberculin, and activation of mononuclear cells, as evidenced by enhanced antitumor, bactericidal, and pyrogenic activities. Recent unpublished work by Damais (personal communication) suggests that some MDP analogs retain adjuvant activity without pyrogenicity. MDP is believed to act via an intermediate target cell, the macrophage, which causes the independent T-cell system to enhance B-cell proliferation (2, 4, 5).

Previous work in our laboratory has shown MDP to protect normal mice challenged with *Klebsiella pneumoniae* either with or without a surgical foreign body (12). Furthermore, MDP protects mice compromised by total starvation, protein deprivation, or cyclophosphamide treatment and enhances the protective effect of a systemic antibiotic (8).

The purpose of the present study was to determine the effect of MDP on *K. pneumoniae* infection in a host experiencing induced hyperferremia, a condition known to be associated with enhanced bacterial infection (13, 18).

**MATERIALS AND METHODS**

**Induced hyperferremia.** Ferric ammonium citrate (FAC) was suspended in distilled water to a concentration of 5,000 µg/ml and autoclaved, and 0.1 ml was injected intraperitoneally (i.p.) into experimental male Swiss Webster mice weighing 20 to 25 g. Simultaneously with the i.p. injection, the mice were challenged with an intramuscular (i.m.) injection of 0.1 ml (9 × 10² to 9 × 10⁵ organisms) of *K. pneumoniae*, capsular type 2, grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). Mice in the control group received injections of bacteria only. Mortality was recorded for a period of 1 week. Statistical analysis was done by computerized probit analysis.

**MDP with induced hyperferremia.** MDP was suspended in sterile saline to a concentration of 5,000 µg/ml, and 0.1 ml (500 µg) was injected subcutaneously into male Swiss Webster mice. After 24 h, the mice...
were challenged with ferric ammonium citrate as described above. Blue 2-0 cotton suture material was impregnated with the *K. pneumoniae* broth (3.03 × 10^8 bacteria per mm of suture) and then was sewn into the gastrocnemius muscle (10). After suture implantation, the mice were killed at intervals. By the use of sterile technique, the gastrocnemius muscle was dissected free and placed in a sterile glass mortar with 5 ml of sterile phosphate buffer solution. The muscle was homogenized for 5 min, and the homogenate was serially diluted in phosphate buffer and plated on nutrient agar (BBL Microbiology Systems). Blood samples were obtained by transecting the major thoracic vessels. The blood samples were serially diluted in phosphate buffer and plated. After the plates were incubated for 18 to 24 h, the colonies were counted. The percentage of bacteria recovered in the muscle and the concentration of bacteria in the blood were determined. Sterile phosphate buffer was prepared by combining 4 g of NaCl, 5 g of K2SO4, 1.5 g of KH2PO4, and 3 g of Na2HPO4 in 1 liter of distilled water, autoclaving, and then adding 1 ml of 1.0 M MgSO4 and 1 ml of 0.1 M CaCl2. Statistical analysis was done by Student’s *t* test (12).

Two other groups of 15 mice were treated with MDP or saline as described above to determine survival and mortality rates. After 24 h, both groups were challenged with an i.p. injection of FAC (as above) and an i.m. injection of 6.6 × 10^8 *K. pneumoniae* organisms in 0.1 ml of sterile phosphate buffer solution. Survival of mice treated with saline or MDP was recorded for a period of 1 week. Statistical analysis was done by the chi-square test with the Yates correction for continuity.

Serum iron determinations were made colorimetrically at four intervals in a separate set of animals receiving MDP or saline over a 6-day period. The number of mice for each treatment and interval was five.

**RESULTS**

**Effect of induced hyperferremia.** Treatment with 500 μg of FAC decreased the 50% lethal dose of mice injected i.m. with *K. pneumoniae* (without suture) approximately 100-fold (2.04 log10 units) compared with control mice. Similarly, the 50% lethal dose of bacteria for the iron-treated mice equaled only the 25% lethal dose of bacteria for control mice (Fig. 1).

No mortality or overt manifestation of iron toxicity was seen in mice receiving 500 μg of FAC without bacteria.

**Effect of MDP on muscle lesions.** Mice with induced hyperferremia that were treated with MDP showed a statistically significant decrease in recovery of bacteria from the homogenate of the infected suture-muscle preparation at 24 and...
Mice were injected intra-peritoneally with MDP or saline alone, for 2 days, and the results are shown in Table 1. The differences between control and treated mice were statistically significant in all experiments. The effect of MDP on serum iron was also examined (Table 1). It was found that the serum iron levels were significantly higher in MDP-treated mice than in controls. The results also indicate that MDP enhances the bactericidal activity of polymorphonuclear leukocytes (PMNs). This is reflected in the prolonged survival of hyperferremic mice given a lethal i.m. injection of K. pneumoniae.

DISCUSSION

The present study shows that induced hyperferremia increases the mortality produced by a bacterial infection and that MDP treatment protects an animal with induced hyperferremia from a local bacterial infection. There are conflicting theories about the mechanism by which iron enhances bacterial infection. Schade and Caroline (15, 16) originally identified transferrin as a serum iron-binding protein that achieves bacteriostasis by withholding iron from the invading pathogens. Other investigators have shown that the normal host responds to infection by decreasing serum iron. This is accomplished by preventing the return of iron from the reticuloendothelial system, which holds over 99% of storage iron (18). This hypoferremia response is mediated by leukocyte-endogenous mediator, a protein produced by activated leukocytes (18). Hypoferremia has also been documented after treatment with endotoxin, M. bovis BCG, and Mycobacterium cell wall extracts (6, 10). However, others (7) found that water-soluble extracts of Mycobacterium cell walls did not produce any change in serum iron or total iron binding capacity at 1 or 24 h after injection.

An alternative explanation for iron enhancement of infection has been proposed according to which iron interferes with cationic proteins within polymorphonuclear leukocytes, preventing intracellular killing of bacteria. Ordinarily, the phagosomes of polymorphonuclear leukocytes contain an iron-binding protein ( lactoferrin) that prevents this interference (1).

The present study confirms that induced hyperferremia is a compromising factor in response to infection, but it does not clarify which of these mechanisms is operant.

MDP provided statistically significant protection from a local bacterial infection in the presence of induced hyperferremia. This result supports the idea that the action of MDP is independent of the alteration of serum iron levels.

Although the differences in levels of bacteremia were not statistically significant, in other experiments (8a), MDP consistently prevented systemic infection by the bacteria from a local infection. This is reflected in the prolonged survival of hyperferremic mice given a lethal i.m. injection of K. pneumoniae.

MDP, a nonspecific enhancer of host defenses, is clearly active against K. pneumoniae in the face of induced hyperferremia. This is the first reported experiment dealing with MDP and hyperferremia. These experiments contribute indirectly to our understanding of the mechanisms of action of MDP and suggest that protection by MDP should be studied for infection-prone patients with abnormalities in their iron metabolism.

LITERATURE CITED