INFECTION AND IMMUNITY, Feb. 1980, p. 288-291
0019-9567/80/02-0288/04$02.00/0

Vol. 27, No. 2

Failure of Synthetic Muramyl Dipeptide to Increase Antibacterial Resistance

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Synthetic muramyl dipeptide, which potentiates antibody production and cellular immune responses at a dosage of 100 to 500 μg, did not enhance resistance to intravenous infection with a sublethal dose of 2×10^3 to 4×10^3 viable *Listeria monocytogenes* cells in mice when intraperitoneally injected either 20 min or 5 days before infection. Similarly, blockade of the mononuclear phagocyte system by dextran sulfate 500 could not be overcome by pretreatment with muramyl dipeptide. In contrast, dextran sulfate 500-induced loss of antibacterial resistance was found to be completely abolished by intraperitoneal injection of 3×10^9 killed *Bordetella pertussis* organisms when given 4 days before injection of dextran sulfate 500, i.e., 5 days before infection. *B. pertussis* were also effective in enhancing antibacterial resistance when administered 5 days before infection. The different behavior of the two adjuvants tested is assumed to be due to their different nonspecific proliferative capacities. Thus, *B. pertussis* are assumed to act by direct stimulation of the mononuclear phagocyte system whereas muramyl dipeptide does not.

The minimal chemical structure for the adjuvant activity of inducing delayed hypersensitivity and enhancing antibody production has been identified as *N*-acetylmuramyl-L-alanyl-D-isoglutamine, a subunit of bacterial cell wall peptidoglycan (1, 7, 14, 15). This muramyl dipeptide, generally referred to as MDP, has recently been synthesized (18) and intensively studied (2, 3, 5, 6, 20). MDP was found to potentiate antibody production and cellular immune responses not only when applied in a water-in-oil emulsion, but also when administered in aqueous solution (6, 20). With soluble protein antigens it proved to be active when given by the parenteral route (3, 6) and devoid of the various side effects known of Freund complete adjuvant (2, 20). More recently, it was learned also that MDP favored the occurrence of anaphylactic reactions to soluble protein antigens in mice (12). However, MDP had only a poor immunopotentiating effect to particulate antigens, such as sheep erythrocytes (12). Little is known concerning the question of whether or not MDP increases antibacterial resistance, which is a characteristic effect of certain immunological adjuvants (4, 10, 21). To fill this gap in information, we carried out the following experiments.

MATERIALS AND METHODS

Mice. Female NMRI mice (specific-pathogen free), weighing 20 to 24 g, were obtained from the Central Institute for Laboratory Animals in Hannover, Germany. All mice were kept under conventional conditions.

Infection. A mouse-virulent strain of *Listeria monocytogenes*, serotype 4b, was cultured in tryptose agar. The virulence of the bacteria used was maintained by continuous passages in mice. The animals were infected with sublethal doses of between 2×10^3 and 7×10^4 viable bacteria, which were injected intravenously (i.v.) in a volume of 0.2 ml of 0.15 M saline.

The mean lethal i.v. dose in young adult NMRI mice was ca. 1×10^4 to 2×10^4 viable bacteria. The experiments were always performed in duplicate assays.

Enumeration of bacteria in spleens of infected mice. The evaluation of resistance to infection with *L. monocytogenes* was made by determining the number of viable organisms present in the spleen at different intervals postinfection. For this purpose, the spleens were removed and homogenized in an Omni-Mixer (Ivan Sorvall, Inc., Newton, Conn.). The homogenate was gradually diluted (10-fold dilution). Each dilution was plated on two replicate dishes with tryptose agar. Colony counts were made after 18 h of incubation at 37°C, and the number of bacteria per spleen was calculated. The significance of the difference of mean values was determined by Student's *t* test.

Adjuvants. MDP and a *Bordetella pertussis* vaccine were used as adjuvants. MDP, a commercial preparation from the Institute Pasteur, Paris, France, was purchased from Fresenius, Homburg, Germany. Doses of either 100, 200, or 500 μg of MDP in 0.2 ml of 0.15 M sterile saline were injected by the intraperitoneal (i.p.) route.

The *B. pertussis* vaccine (OP no. 1207, Behring-Werke, Marburg, Germany) contained 3×10^{10} pertussis organisms (PO) per ml (phase I, not adsorbed, killed at 56°C for 30 min in Merthiolate, 1:10,000). Immediately before injection, the vaccine was diluted

1:2 with 0.15 M sterile saline. A constant volume of 0.2 ml containing 3×10^9 PO was given by the i.p. route. Both MDP and PO were administered either 5 days or 20 min before infection with *L. monocytogenes*.

DS 500. Dextran sulfate 500 (DS 500) (molecular weight, 500,000), purchased from Pharmacia, Uppsala, Sweden, lot no. 7127, was employed to find out whether or not MDP is capable of reversing a DS 500-mediated loss of antibacterial resistance, as regularly produced by pretreatment of mice with PO (10). The activity of the mononuclear phagocyte system was blocked in mice by the injection of DS 500 at a dosage of 50 mg/kg (body weight). All injections of DS 500 were made i.p. in 0.2 ml of 0.15 M sterile saline 24 h before infection with *L. monocytogenes*.

RESULTS

Experiment A. In experiment A, three groups of mice were infected i.v. with 3×10^3 viable *L. monocytogenes* organisms at day 0. The animals of group I, serving as the controls, received no other injection, whereas the other groups of mice were pretreated by an i.p. injection of either 3×10^9 PO (group II) or 100 μg of MDP (group III) 20 min before infection with *L. monocytogenes*.

The control mice (group I) showed the typical course of experimental listeriosis (13, 16, 17), with the maximum number of viable listeriae in the spleens on day 4. Thereafter, a rapid decline occurred. Neither PO nor MDP, when given 20 min before infection, were found to be capable of increasing the resistance of mice to *L. monocytogenes*.

Experiment B. In experiment B, three groups of mice were infected i.v. with 4×10^3 listeriae at day 0. This experiment differed from experiment A in that animals of groups II and III received the i.p. injection of either PO (group II) or MDP (group III) 5 days before infection with *L. monocytogenes*.

The data presented in Figure 1 give clear evidence that pretreatment with PO results in a significant increase of antibacterial resistance in mice. The average numbers of viable listeriae detectable in the spleens of the PO-pretreated mouse group II differed from those of the control group I at all days tested with statistical significance ($2P$ at least < 0.01). In contrast, pretreatment of mice with MDP (group III) was completely ineffective (Fig. 1).

Experiment C. Experiment C differed from experiment B in that mice of the three groups were infected i.v. with 3.6×10^3 listeriae at day 0 and that, furthermore, the mice of group III had received a fivefold-increased amount of MDP (500 μg) 5 days before infection by the i.p. route.

No significant increase in the resistance of mice to *L. monocytogenes* was effected by such a large dose of MDP (group III), whereas a significant increase in antibacterial resistance was produced by pretreatment with PO (group II) (Fig. 2).

Experiment D. To achieve additional information as to whether or not the adjuvants are capable of influencing the course of experimental listeriosis during the early 24-h period, we infected i.v. four groups of mice with 7×10^4 viable *L. monocytogenes* organisms at day 0. The animals of group I, serving as the controls, received no other injection. The other groups of mice were pretreated by an i.p. injection of either 3×10^9 PO (group II), 100 μg of MDP (group III), or 500 μg of MDP (group IV) 5 days before infection with *L. monocytogenes*.

Pretreatment with MDP did not result in any change of antibacterial resistance during the early 24 h after infection, whereas PO effected a significant increase (Fig. 3). Although the colony counts, as determined in the spleens of PO-pretreated mice, were found to be reduced at all

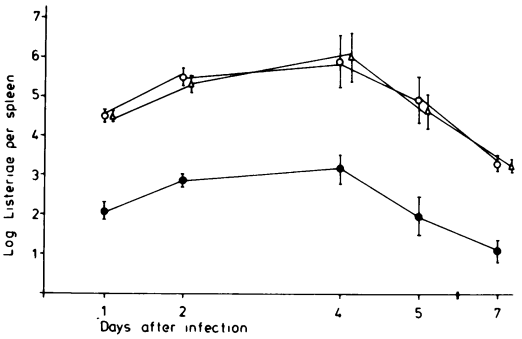

FIG. 1. *Influence of 3×10^9 PO (●) or 100 μg of MDP (△) on antibacterial resistance in mice, when given i.p. 5 days before i.v. infection with 4×10^3 viable listeriae. ○, Controls. Five mice were used per point.*

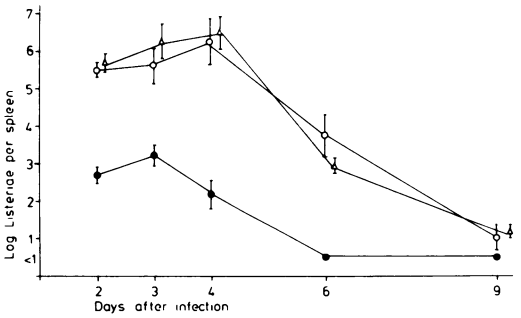

FIG. 2. *Influence of 3×10^9 PO (●) or 500 μg of MDP (△) on antibacterial resistance in mice, when given i.p. 5 days before i.v. infection with 3.6×10^3 viable listeriae. ○, Controls. Five mice were used per point.*

times tested, only the mean values determined 4 h ($2P < 0.005$) and 24 h ($2P < 0.002$) after infection differed with statistical significance from those of the corresponding control group I.

Experiment E. In experiment E, four groups of mice were infected i.v. with 2×10^3 listeriae at day 0. While the mice of group I, serving as the controls, received no further treatment, the other three groups received, 24 h before infection, an i.p. injection of 50 mg of DS 500 per kg. Additionally, mice of groups III and IV had been pretreated by an i.p. injection of either 3×10^9 PO (group III) or 200 μg of MDP (group IV) 4 days before treatment with DS 500, i.e., 5 days before infection with *L. monocytogenes*.

All animals of the DS 500-treated group II died within the initial 3-day period postinfection (Fig. 4). The same applied to the animals of group IV pretreated with 200 μg of MDP 4 days before the injection of DS 500 (Fig. 4). In contrast, all mice of group III pretreated with 3×10^9 PO survived. In this way the numbers of viable listeriae detectable in the spleens at specific intervals postinfection were found to be reduced, as compared to the corresponding values of the control group I (Fig. 4). This evidently shows that, in contrast to the impressive activity of PO, DS 500-induced loss of antibacterial resistance is not influenced by pretreatment with MDP.

DISCUSSION

Numerous agents known as effective immunological adjuvants are capable of enhancing the nonspecific resistance of the host to infection and tumors (cf. 4). As reported previously (10, 21), this also applies to PO. However, the simultaneous administration of PO (i.p.) and *Listeria* (i.v.) did not promote increased resistance (10).

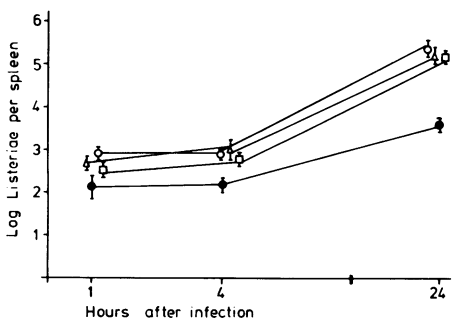

FIG. 3. *Influence of either 3×10^9 PO (●), 100 μg of MDP (□), or 500 μg of MDP (△) on antibacterial resistance in mice when given i.p. 5 days before i.v. infection with 7×10^4 listeriae. ○, Controls without any pretreatment before infection. Five mice were used per point.*

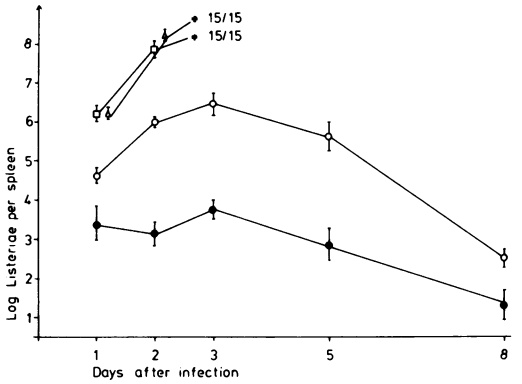

FIG. 4. *Influence of 3×10^9 PO or 200 μg of MDP on DS 500-mediated loss of antibacterial resistance. Symbols: ○, i.v. infection with 2×10^3 listeriae at day 0; □, i.v. infection with 2×10^3 listeriae at day 0 after i.p. pretreatment with DS 500 at day −1; △, i.v. infection with 2×10^3 listeriae at day 0 after i.p. pretreatment with both MDP at day −5 and DS 500 at day −1; ●, i.v. infection with 2×10^3 listeriae at day 0 after i.p. pretreatment with both PO at day −5 and DS 500 at day −1. Five to six mice were used per point.*

Such activity became demonstrable only when PO were given several days before infection with *L. monocytogenes*. The optimal interval was found to be 5 to 15 days, although a significant degree of protection was still detectable when PO were given as early as 45 days before infection (10). The data reported here, as documented in Fig. 1 to 3, are in full accord with our previous findings. In contrast, synthetic MDP, which potentiates antibody production and cellular immune responses (6, 20), did not show any influence on antibacterial resistance, even though an extremely large dose of 500 μg of MDP was administered (Fig. 2 and 3). Blockade of the mononuclear phagocyte system by DS 500 virtually eliminates the mechanisms of defense against infection by *L. monocytogenes* (8, 10, 11), resulting in overwhelming necropurulent inflammatory processes and death of the animals, due to the elimination of a cell system that plays a fundamental role in the defense against intracellular parasites, such as *L. monocytogenes* (cf. 10). However, such lethal effects of DS 500 can be completely abolished by pretreatment of animals with PO. Treatment not only increases survival rates from 0 to 100% (Fig. 4), but also reduces morphological changes to a level more or less identical to that of the controls (10). MDP was found to be completely ineffective in overcoming DS 500-induced loss of antibacterial resistance (Fig. 4).

It is generally believed that the capacity to

enhance nonspecific resistance of the host to infections and tumors is effected by a direct stimulation of the mononuclear phagocyte system (4, 10, 21) or by modulation of phagocyte function by means of lymphokines (4) or by both stimulation and modulation. PO evidently function as a nonspecific proliferative impulse (cf. 9). Therefore, we are inclined to favor the view that a PO-mediated increase in antibacterial resistance is mainly due to a direct stimulation of macrophages. This assumption is substantiated by the observation that the amount of DS 500 detectable in histological sections of the liver and spleen of mice injected with PO in addition to DS 500 was significantly smaller than that in animals receiving DS 500 only (10). Taking into account that MDP does not possess nonspecific proliferative activity (19), it becomes clear why MDP was found to be neither capable of increasing antibacterial resistance nor of overcoming a DS 500-induced loss of antibacterial resistance.

ACKNOWLEDGMENT

This paper was supported by Bundesmimsterium des Junern.

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