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Enhancement of the local inflammatory response to bacterial infection by muramyl dipeptide

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Summary. The effect of the synthetic immuno-adjuvant compound, muramyl dipeptide (MDP), upon the local inflammatory response to experimental bacterial infection was assessed by histological examination. Within 24 h of the insertion of a bacteria-laden suture into the medial thigh musculature of mice treated with either MDP or placebo, an enhanced degree of polymorphonuclear leucocyte infiltration in the muscle around the suture was observed in the MDP-treated animals. The inflammatory response around a sterile suture was less intense in both treatment groups and specific correlation between the degree of local inflammation and the extent of bacteraemia developing in either group of animals was not noted. The previously observed protection conferred by MDP against the local impact of bacterial challenge appears to be mediated in part by enhancement of the acute local inflammatory response.

Keywords: muramyl dipeptide, local inflammatory response, bacterial infection

Surgical infection continues to be a clinical problem despite meticulous attention to sterile technique and the increasingly appropriate use of antibiotic prophylaxis. Recently there has been a resurgence of interest in the stimulation of non-specific host defenses as an additional means by which surgical, as well as other, infection may be controlled (Polk & Galland 1982). The isolation and subsequent synthesis of muramyl dipeptide (MDP), the minimum structural component of the mycobacterial cell wall in Freund's complete adjuvant, has provided an agent seemingly remarkably free from the imperfections with regard to timing of administration and side-effects which were seen with previous immuno-adjuvant agents (Chedid & Audibert 1977). In addition, MDP has been shown to protect experimental animals from the effects of a wide variety of bacterial challenges and host defense impairments (Polk & Galland 1982).

A consistent observation with MDP treatment in previous experiments with simulated surgical infection has been a reduction in the percentage of bacteria recovered from the local implantation site within 24 h of a bacterial challenge (Galland & Polk 1982). Although the protection which is conferred against subsequent bacteraemia and mortality has been an even more impressive feature of MDP treatment, much of this systemic protection may arise as a result of enhanced local control and containment of the bacterial challenge (Polk & Galland 1982). In order to further define the mechanisms by which MDP exerts these protective effects, the inflammatory response in the tissues surrounding a suture impregnated with *Klebsiella pneumoniae* has been

graded by histological examination at timed intervals following the insertion of the suture into the hind leg musculature of mice treated with MDP or placebo. In addition, the relationship between the degree of inflammation observed locally and the extent of subsequent bacteraemia has been examined.

The suture challenge has been well characterized (Fagelman *et al.* 1981) and represents all of the elements of a surgical infection, namely tissue damage, bacteria and a foreign body. The test organism, *Klebsiella pneumoniae*, is a representative Gram-negative organism of surgical significance which is characterized by its ability in this model to overwhelm the host defenses and result in an ultimately fatal bacteraemia in a majority of the placebo-treated test animals (Polk & Galland 1982). It was anticipated that the suture itself, acting as a foreign body, would induce an inflammatory response independent of the bacterial challenge. Consequently animals receiving a sterile suture were also studied for control purposes.

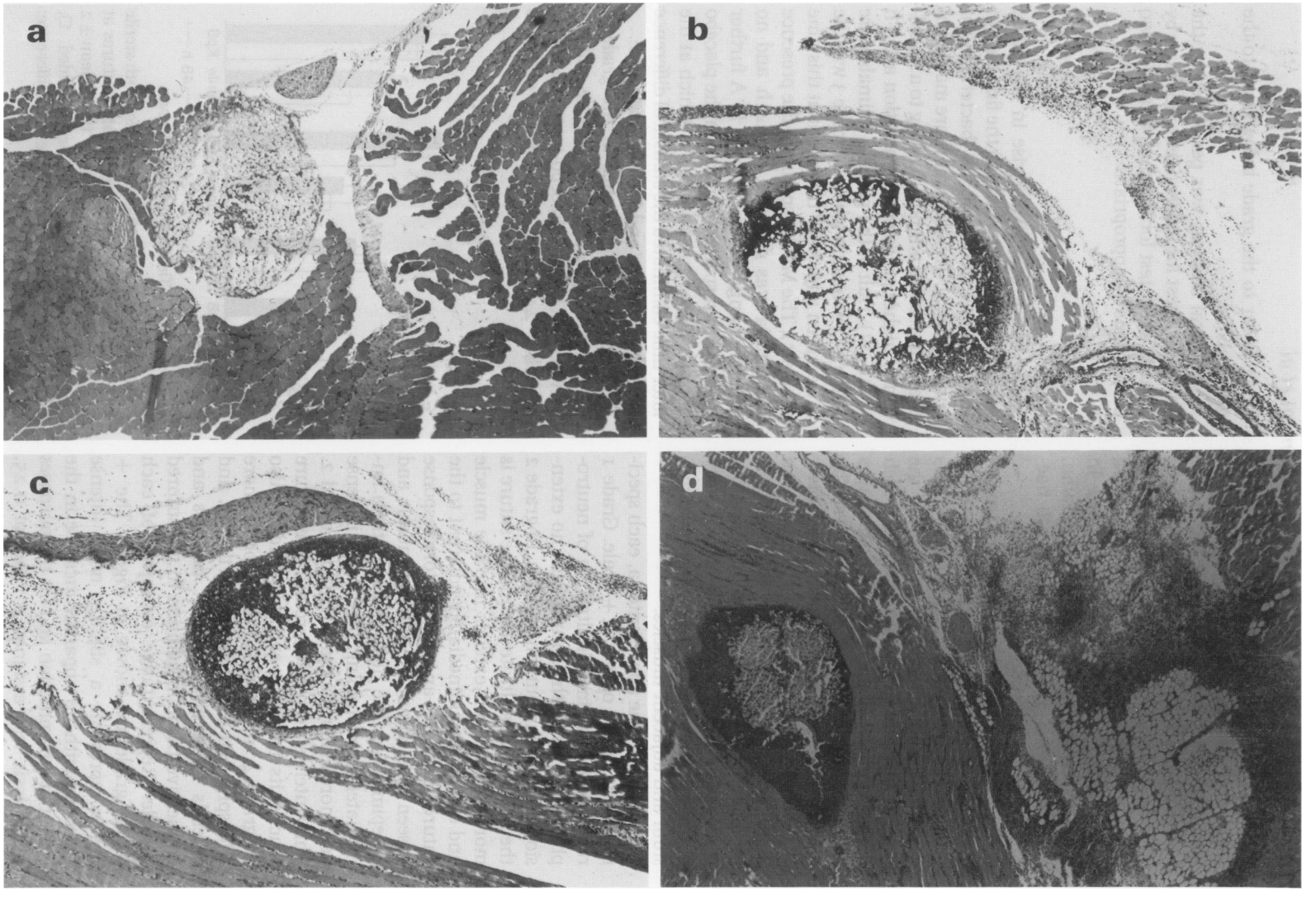
Materials and methods

Adult male Swiss Webster mice weighing between 25–35 g were used throughout these experiments. Twenty-four hours before receiving the suture challenge, the animals were randomized to receive either 100 μg of muramyl dipeptide (Groupement d'interet Economique-institut pour la Recherche et la Production d'Immunostimulants, GIRPI, Paris, France) or an equal volume of its solvent, phosphate-buffered saline, subcutaneously on to their backs. The subcutaneous route of administration was used for convenience, although MDP is equally effective whether given by the oral, subcutaneous, intramuscular or intravenous routes (Chedid *et al.* 1977).

Lengths of 2–0 cotton suture were immersed in trypticase soy broth (BBL, Cockeysville, MD) and sterilized in an autoclave. The broth was then incubated at 37°C for 18 h after it had either been inoculated with the test organism (*Klebsiella pneumoniae* capsular Type 2 [KpB]) to provide bacteria-impregnated sutures or left undisturbed to provide sterile sutures. Lengths of suture attached to a French eye needle were then inserted aseptically into the medial right thigh musculature of the mice and the suture was cut flush with the skin at both ends. Other known lengths of suture were placed in 5 ml of phosphate buffered saline and homogenized using a sterile glass mortar and electrically driven teflon pestle. This homogenate underwent quantitative bacterial analysis by serial dilution, plating on trypticase soy agar (BBL, Cockeysville, MD) and overnight incubation at 37°C, both to determine the dose of *Klebsiella pneumoniae* per millimeter of suture and to confirm the absence of contamination of the sterile sutures. In these experiments, the average dose of bacteria was 3.1×10^5 organisms per millimeter of suture.

There were four study groups which comprised animals receiving: a, MDP and a *K. pneumoniae*-laden suture; b, placebo and a *K. pneumoniae*-laden suture; c, MDP and a sterile suture; and d, placebo and a sterile suture. At 3, 6, 24 and 48 h following the insertion of a bacteria-laden suture, 10 MDP-treated and 10 placebo-treated animals were killed by cervical dislocation. In addition, 10 animals from each sterile suture group were killed at 24 and 48 h. Blood was obtained by cardiac puncture and the extent of bacterae-

Fig. 1. Photomicrographs of the mouse hind leg musculature containing the suture showing the major grades of inflammation. (*a*) Grade 1. Minimal infiltrate of neutrophils within the suture. Essentially no inflammatory response within the adjacent skeletal muscle. (*b*) Grade 2. There is more intense inflammatory infiltrate around the suture with minimal extension into the surrounding muscle and connective tissue. (*c*) Grade 3. The inflammatory response is greater around the suture and there is more extension into the muscle and connective tissue. (*d*) Grade 4. Maximal inflammation extending into surrounding muscle and connective tissue.


a
b
c
d

mia was quantified by serial dilution in sterile saline and plating on trypticase soy agar. The medial muscle mass containing the suture was excised 'en bloc' from the right thigh and fixed by immersion in 10% buffered formalin. Subsequently, the muscle was subdivided into 1.5-mm-thick blocks, dehydrated in graded alcohols and embedded in paraffin. Sections of 6–8 μm were stained with haematoxylin and eosin.

The intensity and extent of the inflammatory response were subjectively evaluated by an observer from whom the treatment status was specifically concealed, as well as the sample time and the suture group to which each specimen belonged. Each specimen was therefore examined blind. In the analysis the following morphologic features were noted: 1, Intensity of the inflammatory response in and immediately adjacent to the suture; 2, Intensity and extent of the inflammatory response in the sutured muscle; 3, Intensity of the inflammatory response in the epimysium and adjacent muscles.

Based upon the above criteria, each specimen was graded on a 1–4+ scale. Grade 1 represents a minimal infiltration of neutrophils confined to the suture with no extension into the surrounding tissues. In grade 2 the neutrophil infiltrate around the suture is more marked and extends into the muscle and epimysium immediately adjacent to the suture. In grade 3 an inflammatory response is seen throughout the sutured muscle and its epimysium; the neutrophil infiltrate immediately surrounding the suture is dense and more extensive than in grades 1 and 2. This intense response around the suture reaches its maximum extent in grade 4. Also in grade 4, neutrophil infiltration is more widespread in the surrounding tissues, and extends to involve adjacent muscles and connective tissue in addition to the sutured muscle. Representative examples of each major grade are shown in Fig. 1. The + grade represents a slightly more intense response with no apparent increase in the extent of inflammation and for the purposes of analysis was assigned a value of 0.25, being closer to the grade below than to the grade above.

Statistical analysis was performed by the Student's *t*-test for independent means or by Fisher's exact test for the comparison of two proportions as appropriate.

Results

There was a progressive rise in the mean grade of inflammation during the first 24 h in the animals receiving a bacteria-laden suture (Fig. 2). This rise was more marked in the MDP-treated animals, leading to a significantly higher grade of inflammation at 24 h ($P<0.05$). Half of the placebo animals were grade 2–2+ at 24 h whereas grade 3 was the lowest seen with MDP treatment at this time (Table 1). The MDP response in the presence of bacteria was maximal by 24 h and no further change was seen at 48 h. A further increase in response occurred in the placebo animals between 24 and 48 h, which at the later time eliminated the statistical difference between placebo- and MDP-treated mice.

There was no difference in the grade of inflammation around a sterile suture between MDP- and placebo-treated animals at either 24 or 48 h, although only the

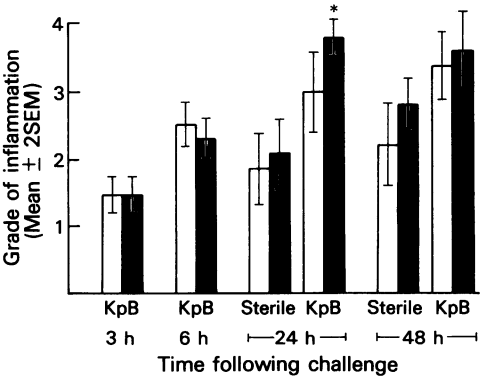


Fig. 2. Mean grade of inflammation for both sterile and *K. pneumoniae* (KpB)-impregnated sutures at the different study times. Note that mean ± 2 standard errors of the mean are provided. □, Placebo; ■ MDP. Significance of difference: *$P<0.05$.

Table 1. Number of animals with each grade of inflammation at the 24-h sample time. (*K. pneumoniae* suture)

Grade of inflammation	Placebo	MDP
1–1+	0	0
2–2+	5*	0
3–3+	1	3
4–4+	4	7

Significance of difference:
*$P<0.05$, Fisher's exact test.

MDP-treated animals showed a significant rise in grade between the two sample times ($P<0.05$). The presence of *Klebsiella pneumoniae* on the suture induced a significantly greater inflammatory response in both the MDP ($P<0.01$) and placebo ($P<0.02$) groups at 24 h when compared to their respective sterile suture groups. These differences persisted at 48 h in both the MDP ($P<0.05$) and placebo ($P<0.01$) groups.

MDP conferred significant protection against bacteraemia at 6, 24 and 48 h ($P<0.01$) compared to the placebo group (Fig. 3). Although the difference was most marked at 24 h, there were no significant correlations between the grade of inflammation and the degree of bacteraemia at any of the sample times (Table 2). Bacteraemia did not occur in the animals receiving a sterile suture.

Discussion

The concept of host defense stimulation is an old one predating, but overshadowed by, the advent of antibiotic therapy. Much of the early work in this field has been hindered by problems relating to dosage, timing of administration and side-effects with many immuno-adjuvant agents (Chedid *et al.* 1978). In this context, MDP represents a considerable improvement upon its predecessors, being effective over a wide dose range whether given 4 days or 1 h before the bacterial challenge (Chedid *et al.* 1977) and with little or no significant side-effects thus far (Wachsmuth & Dukor 1981). Furthermore, the appreciation that appropriate antibiotic therapy may not always successfully control infection, particularly in the im-

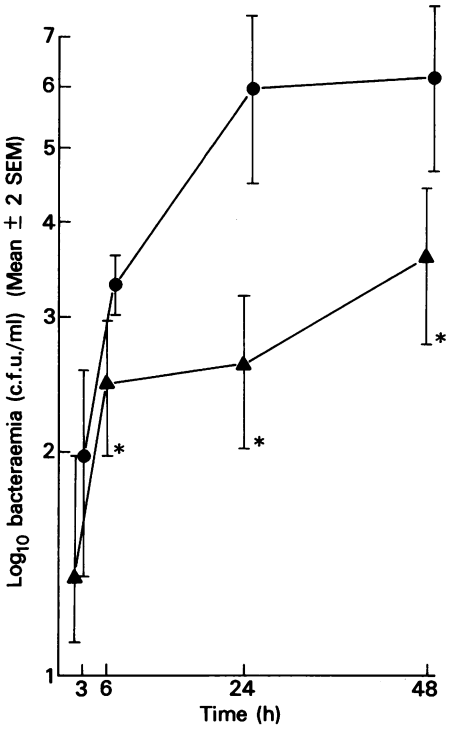


Fig. 3. Extent of bacteraemia following the implantation of a *K. pneumoniae* laden suture. ●, Placebo; ▲, MDP. Significance of difference: *$P<0.01$.

Table 2. Correlation coefficients (r) between grade of inflammation and bacteraemia at the various sample times

Time (h)	Placebo	MDP
3	0.20	0.17
6	0.31	0.20
24	−0.42	−0.08
48	0.10	0.12

munocompromised host (Bakker-Woudenberg *et al.* 1979; Bodey 1978), has served to emphasize the considerable promise which host defense stimulation holds as an approach additional to antibiotics in the prevention and management of infection (Polk *et al.* 1982). In order to better define the potential clinical applications of such immuno-adjuvant agents, it is essential to understand the mechanisms by which they exert protective effects against infectious challenges.

There is an accumulating body of evidence to implicate the macrophage as a primary target cell of MDP (Fevrier *et al.* 1978). Known effects include the activation of peritoneal macrophages (Nagao *et al.* 1979), stimulation of the release of T cell activating factors by macrophages (Iribe *et al.* 1981) and increased phagocytic activity of the reticulo-endothelial system (Tanaka *et al.* 1979). The effects of MDP upon polymorphonuclear leucocyte (PMN) function are less well established, and although MDP appears to enhance PMN phagocytosis of *Eschericia coli*, this effect is not seen with an encapsulated strain of *K. pneumoniae* (Osada *et al.* 1982) such as the one used in these experiments. It would seem, therefore, that MDP-induced enhancement of PMN phagocytosis is not a factor in the consistent reduction in local bacterial recovery which has been observed 24 h following the insertion of a *K. pneumoniae*-laden suture.

The results of these histologic studies, however, do suggest that MDP induces an accelerated local inflammatory response, resulting in a greater extent and intensity of leucocyte infiltration in the muscles around the suture by 24 h following the bacterial challenge. This effect appears to result from the presence of bacteria rather than from the suture *per se* (Fig. 2) and this is supported by the observation that suppression of the inflammatory response by the prior administration of a large dose of hydrocortisone sodium phosphate abolishes the local impact of MDP (Galland *et al.* 1983). Likewise, subsequent work in this laboratory suggests that MDP enhances PMN chemotaxis *in vitro* (P.M. Lamont, unpublished observations) consistent with the histological observations in this study.

Treatment with MDP induces a dramatic reduction in the degree of bacteraemia seen in this model (Fig. 3), in comparison to its more modest local impact. It has been suggested that this systemic control may result partly from the prevention of bacterial dissemination into the bloodstream by MDP (Polk & Galland 1982), and certainly an intense tissue leucocytosis has traditionally been presumed to be a barrier to the further spread of infection (Miles 1980). However, we did not detect a specific correlation between the intensity of the local inflammatory response and the control of subsequent bacteraemia in individual animals.

While the weight of evidence continues to favour a direct or mediated effect upon the macrophage as the elusive mechanism of action, these studies conclusively demonstrate that specific intense local accumulation of polymorphonuclear leucocytes is a part of the MDP-mediated response to local bacterial challenge.

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