Br. J. exp. Path. (1987) **68**, 655–661

Stimulatory effects of muramyl dipeptide upon neutrophils isolated from a local bacterial infection

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Received for publication 24 March 1987
Accepted for publication 4 April 1987

Summary. This study examined the effects of muramyl dipeptide (MDP) *in vivo* upon the local inflammatory response to a bacterial challenge. In addition to quantitative bacteriology of the tissues surrounding an infected suture, polymorphonuclear leucocytes (PMN) involved in the local inflammatory response were extracted and estimations made of their number, viability and phagocytic activity. Fewer bacteria were recovered from the muscle around the suture in MDP-treated animals compared to placebo-treated controls ($P<0.02$), although there was no difference in the number of bacteria on the suture itself. Polymorphonuclear leucocytes were present in greater numbers ($P<0.01$), more PMNs were viable ($P<0.01$) and more PMNs had visibly phagocytosed bacteria ($P<0.01$) in the MDP group compared to the placebo group. These data indicate that MDP enhances the local inflammatory response to infection with increased influx, viability and phagocytic activity of PMNs, resulting in improved local control of a test bacterial challenge.

Keywords: muramyl dipeptide, neutrophils, bacterial challenge

Infection remains a major factor limiting the recovery of patients after injury or operation (Cruse & Foord 1980). Increasing recognition of the limitations of antisepsis, asepsis and antibiotic therapy has re-awakened interest in the stimulation of nonspecific host defences as an additional means of controlling bacterial infection (Chedid *et al.* 1978; Polk & Galland 1982). Muramyl depeptide (MDP) is a water soluble peptidoglycan derived from the mycobacterial cell wall (Ellouz *et al.* 1974) and its protective effects against experimental bacterial infection have been clearly demonstrated (Chedid *et al.* 1978). MDP is the minimal active component of Freund's complete adjuvant and appears to be free of the difficulties with timing of administration and side-effects seen with previous immunoadjuvent agents (Chedid *et al.* 1977).

The precise mechanisms by which MDP exerts its protective effects remain incompletely understood. While the macrophage has been implicated as a major target cell (Fevrier *et al.* 1978), polymorphonuclear leucocytes (PMN) also play a predominant role in the early phases of host defence against many acute soft tissue infections. A previous histological study has indeed shown increased numbers of PMNs around a *Kleb-*

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siella pneumoniae impregnated suture in MDP-treated mice 24 h following insertion of the suture (Lamont *et al.* 1984).

Analysis of the effects of MDP upon PMNs is complicated by differences both in the functional characteristics and in the relative proportions of neutrophil subsets which exist between samples of circulatory and exudative PMNs (Klempner & Gallin 1978; Wandall 1982). The aim of this study was to assess in more detail the effects of MDP upon the exudative PMNs *directly* involved in the local inflammatory response to bacterial infection in an attempt to further understand the previously noted histological findings.

A well-established model of surgical infection was used, involving the insertion of a bacteria-laden suture into the mouse thigh (Polk *et al.* 1981). This model represents the features of an infected wound, namely tissue trauma, bacterial contamination and a foreign body. *Klebsiella pneumoniae* was used as a representative gram negative organism of surgical significance against which the protective effects of MDP are well established (Chedid *et al.* 1978). Quantitative bacteriology from the suture and the surrounding thigh musculature was undertaken in combination with attempts to measure the influx, viability and phagocytic activity of PMNs surrounding the infected suture. In anticipation that the suture itself would induce an inflammatory response independent of the bacterial challenge, control studies were also performed using a sterile, uncontaminated suture.

Materials and methods

Adult male Swiss Webster mice weighing 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, (Groupment d'interet Economique-institut pour la Recherche et la Production d'Immunostimulants, Paris, France) or an equal volume of its solvent, phosphate buffered saline (PBS), subcutaneously on to their backs.

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 been either innoculated with *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 either end. Other known lengths of suture were homogenized and underwent quantitative bacterial analysis by serial dilution, plating on trypticase soy agar (BBL, Cockeysville, MD) and overnight incubation at 37°C to both determine the dose of *K. pneumoniae* per millimetre of suture and to confirm the absence of contamination of the sterile sutures. In these experiments the average dose of *K. pneumoniae* was $3{\cdot}6 \times 10^5$ $\pm 0.34 \times 10^5$ organisms per millimetre of suture. There were four study groups comprised of animals receiving the following: (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 each interval of 6, 24 and 48 h following insertion of the suture, 10 animals from each group were killed by cervical dislocation. The medial thigh musculature containing the suture was excised. The suture was then extracted and placed immediately in 0.2 ml of minimal essential medium (MEM) (Gibco, Grand Island, NY), where it was gently agitated for precisely 1 min to wash off the cells and bacteria adhering to the suture. The resulting suspension was then divided for analysis as follows.

PMN viability. Ten microlitres of suspension was immediately placed on a glass slide and mixed with an equal volume of trypan blue solution. A glass coverslip was applied and PMN viability determined by counting 100 PMNs in continguous high power fields and recording the percentage able to exclude the dye. The slides were coded so that the

treatment groups were not known to the person reading the sample.

PMN phagocytic activity. A further 10 μl of suspension was placed on a glass slide, fixed in methanol and stained with Giemsa stain. The slides from each group were then assessed for the percentage of PMNs that had visibly phagocytosed *K. pneumoniae* by an observer who was specifically not aware of the treatment group to which each slide belonged. One hundred PMNs in contiguous fields were examined under oil immersion for the presence and number of *K. pneumoniae* lying within their cytoplasm. The bacteria were easily seen due to the unstained zone produced around them by their capsule, although it was not possible to determine which of the PMNs containing bacteria remained viable.

PMN numbers. The number of PMNs present around the suture was quantified after dilution of the suspension in leucocyte counting fluid. The PMN's were counted in a Neubauer chamber, their total number of the original 0.2 ml of suspension was calculated, and the results were expressed as the number of PMNs per millimetre of suture extracted from the muscle.

Bacterial recovery from the suture. A 0.1 ml aliquot of the suspension was serially diluted in sterile PBS, plated on trypticase soy agar and incubated overnight at 37°C. The number of colony forming units was then counted to quantitate the number of *K. pneumoniae* on the suture. Suture length was also measured with a micrometre gauge and from this the original dose of bacteria administered to each animal was calculated by reference to the known number of bacteria initially present on the suture (3.5×10^5/mm). Results were then expressed as the percentage of bacteria recovered from the suture compared to the dose originally administered, the percentage being converted to natural logarithms for clarity of presentation.

Bacterial recovery from the muscle. The right medial thigh musculature, all of which had been excised from each animal, was suspended in 5 ml of PBS and homogenised using a sterile glass mortar and an electrically-driven, sterile teflon pestle. The homogenate was serially diluted in PBS, plated on trypticase soy agar and incubated overnight at 37°C. From the number of colony forming units present, the total number of Klebsiella in the muscles around the suture was calculated, and the results again expressed as the logarithm of the percentage of bacteria recovered compared to the dose originally administered on the suture.

Statistical analysis. All results underwent statistical analysis using the Student's *t*-test for independent means.

Results

Bacterial recovery

There were consistently fewer *K. pneumoniae* in the muscles around the suture in the MDP group compared to placebo animals (Table 1), although this difference achieved statistical significance only by 48 h after the challenge ($P<0.02$). There were no differences however, in the numbers of bacteria recovered from the suture itself between the two treatment groups.

PMN numbers

The number of PMNs recovered from the surface of the suture were remarkably similar between MDP and placebo treated animals at 6 h and 24 h after the bacterial challenge (Fig. 1). By 48 h, however, there were almost twice as many PMNs around the suture in MDP animals compared to placebo animals ($P<0.01$).

Following the insertion of a sterile suture, the number of PMNs present at 6 h and 24 h were below the limits of detection in both groups (i.e. $<2 \times 10^4$/ml of suspension). At 48 h there were more PMNs recovered from

Table 1. Percentage recoveries from the suture and from the thigh muscles of MDP and placebo treated mice (mean ± 2 s.e.m.)

		Hours following challenge		
Log_{10}% *K. pneumoniae*		6	24	48
Suture recovery	MDP	2.5±0.3	2.6±0.3	2.8±0.2
Suture recovery	Placebo	2.7±0.2	2.8±0.3	2.8±0.3
Muscle recovery	MDP	2.6±0.3	3.0±0.2	2.8±0.2
Muscle recovery	Placebo	3.0±0.1	3.2±0.2	3.4±0.4*

* $P<0.02$.

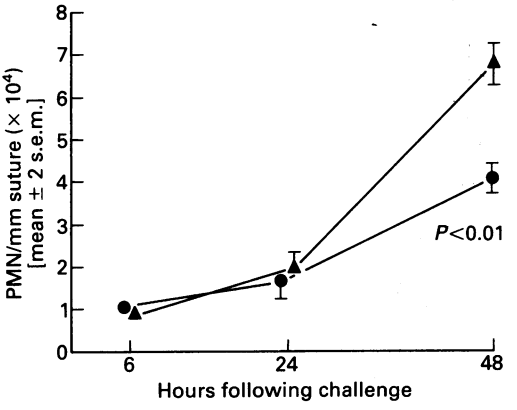

Fig. 1. Number of PMNs × 10^4 per unit length of suture in animals receiving a bacterial challenge. ▲, MDP; ●, placebo.

the sterile sutures in MDP animals ($8.5 \times 10^3 \pm 1.8 \times 10^3$ PMN/mm suture) than from the placebo animals ($3.2 \times 10^3 \pm 1.0 \times 10^3$ PMN/mm suture). This finding represented a significant difference ($P<0.01$) which closely mirrored the findings with bacteria-laden sutures, although there were 10-fewer PMNs around the sterile sutures.

PMN viability

There was little change in PMN viability around a sterile suture over the study period (Table 2) and there were no differences between MDP or placebo treated animals. There was however, a progressive decline in the percentage of viable PMNs around the *K. pneumoniae* suture in the placebo animals

Table 2. Percentage of viable PMNs washed off the suture at timed intervals following its insertion (mean ± 2 s.e.m.)

		Hours following challenge		
Per cent viable PMNs		6	24	48
Sterile suture	MDP	89±4	88±3	89±4
Sterile suture	Placebo	89±3	86±3	84±4
K. pneumoniae suture	MDP	91±2	92±2	90±1
K. pneumoniae suture	Placebo	91±3	81±4**	78±4**

** $P<0.01$.

Table 3. Phagocytosis of *K. pneumoniae* by PMNs washed off the suture in MDP and placebo treated mice (mean ± 2 s.e.m.)

		Hours following challenge		
		6	24	48
PMN containing *K. pneumoniae* (%)	MDP	3.5 ± 1.6	2.6 ± 0.9	3.8 ± 1.4**
PMN containing *K. pneumoniae* (%)	Placebo	2.1 ± 1.6	4.2 ± 1.4	1.4 ± 0.7**
K. pneumoniae phagocytosed (%)	MDP	0.11 ± 0.09	0.09 ± 0.05	0.27 ± 0.13**
K. pneumoniae phagocytosed (%)	Placebo	0.03 ± 0.02	0.09 ± 0.06	0.11 ± 0.07**

**$P < 0.01$.

which did not occur in the MDP treated animals, with significant differences between the two groups at both 24 h and 48 h (Table 2).

PMN phagocytic activity

At 6 h and 24 h there were no significant differences between the phagocytic activity of PMNs around the suture from MDP animals compared to placebo animals (Table 3); the mean percentage of PMNs containing *K. pneumoniae* ranged from 2 to 4% of the total. In the placebo animals at 48 h however, fewer than 2% of the PMNs contained bacteria, and this was significantly lower than in the MDP animals ($P < 0.01$).

There were no differences observed between MDP and placebo in the mean number of *K. pneumoniae* within each PMN. Extrapolating from the total number of bacteria phagocytosed per 100 PMN, however, estimates were made of the percentage of bacteria which had been phagocytosed according to the following formula:

$$\frac{\text{number of bacteria phagocytosed per PMN} \times \text{number of PMN around suture}}{\text{bacterial recovery from the suture}} \times 100$$

Again there were no differences in the percentage of bacteria phagocytosed at 6 h and 24 h (Table 3), but by 48 h a significantly higher percentage of *K. pneumoniae* had been phagocytosed in the MDP group ($P < 0.01$).

Discussion

The potential benefits of nonspecific host defence stimulation have long been appreciated. Clinical application of this principle has been hampered by difficulties with timing of administration and side-effects with each successive immunoadjuvent agent. The isolation and synthesis of muramyl dipeptide represents a promising step toward the provision of a reliable and seemingly safe agent. In order to better define the potential clinical role of MDP, it is important to understand the mechanisms by which it exerts protective effects against infection.

An accumulating body of evidence had implicated the macrophage as a target cell of MDP (Lowy *et al.* 1977). 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), enhanced release of superoxide anion by peritoneal macrophages (Cummings *et al.* 1980) and increased phagocytic activity of the reticuloendothelial system (Tanaka *et al.* 1979). The effects of MDP upon PMN function are less well established. Histological examinations of sections cut from the muscles around an infected suture have demonstrated a more intense polymorphonuclear response in mice treated with MDP (Lamont *et al.* 1984). Suppression of the acute inflammatory response, on the other hand, by large doses of

hydrocortisone sodium phosphate, abolishes the locally protective effects of MDP against a bacterial challenge (Galland *et al.* 1983).

In the present study, increased numbers of PMNs were washed off the infected suture at 48 h in the MDP animals. This finding suggests either reduced adherence of PMNs to the suture or increased influx of PMNs around the suture. The latter mechanism is consistent both with the previous histological findings (Lamont *et al.* 1984) and with the observed enhancements of in-vitro PMN chemotactic activity produced by prior incubation of PMNs with MDP (Brown *et al.* 1985). Indeed, an intense tissue leucocytosis has traditionally been considered to act as a barrier to the spread of bacteria from an infective focus (Miles 1980). Quantitative bacteriological data from the present study demonstrate that whereas the same number of bacteria are present on the suture at 48 h in both the MDP and placebo treated animals, significantly fewer bacteria are present in the muscles around the suture in the MDP group (Table 1).

Increased numbers of PMNs were also recovered from the sterile sutures in MDP treated animals at 48 h and this effect is not therefore related specifically to the *K. pneumoniae* challenge. Enhancement of PMN viability by MDP, on the other hand, occurs only in response to the bacterial challenge and is not seen around the sterile suture (Table 2). The precise mechanism by which MDP exerts this effect is unclear on the basis of this study, but may reflect either reduced susceptibility of the PMNs to the toxic effects of the *K. pneumoniae* challenge or enhanced macrophage clearance of damaged PMNs. This effect of MDP upon PMN viability has not been previously reported and requires to be taken into account in any future functional studies.

Muramyl dipeptide also enhanced PMN phagocytosis of *K. pneumoniae* at 48 h, both in terms of the percentage of bacteria which had been phagocytosed and the percentage of PMN containing bacteria. This finding contradicts that of Osada *et al.* (1982) who were unable to demonstrate such an effect following intraperitoneal challenge with a similarly encapsulated strain of *K. pneumoniae*. In the latter study however, the maximum time from the challenge to the extraction of PMNs for phagocytic assay was only 2 h, and quantitative bacteriology of the peritoneal exudate was not provided. The protective effects of MDP against the rather stringent bacterial challenge in the present study are only apparent after 48 h (Table 1), coinciding with the enhancement of PMN phagocytic activity. The proportion of PMNs containing bacteria, however, remains very small at less than 5%, reflecting the resistance of encapsulated strains of *K. pneumoniae* to PMN phagocytosis (Fukutome *et al.* 1980).

Although considerable evidence exists to implicate a direct or mediated effect upon the macrophage as the primary mechanism of its action, these studies demonstrate that MDP actively promotes the influx, viability and phagocytic activity of PMNs involved in the acute inflammatory response, contributing to improved local control of a bacterial challenge.

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