

# Enhancement in anti-Semliki Forest virus activity of ds RNA by a muramyl dipeptide

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Antiviral activity of an interferon-inducing mycoviral ds RNA against Semliki Forest virus infection was considerably enhanced by *N*-palmitoylmuramyl-L-alanyl-D-isoglutamine (PMDP), a new muramyl dipeptide. This enhancement in activity was not due to increased production of interferon, but resulted probably from a PMDP-induced increase in nonspecific resistance to infection. These results indicate that a combined treatment with an interferon inducer and muramyl dipeptide may prove highly useful to control effectively viral infections.

*Viral infection    Interferon inducer    Muramyl dipeptide    Macrophage activation*

## 1. INTRODUCTION

MDP and its derivatives stimulate humoral and cell-mediated immune responses to various antigens [1,2] and are known to enhance nonspecific resistance to bacterial, fungal and protozoal pathogens [3–5]. In addition, treatment of mice with liposomes containing muramyl tripeptide has been shown to confer protection against herpes simplex type 2 infection [6]. Here we demonstrate that the antiviral activity of an interferon-inducing mycoviral ds RNA is considerably enhanced if the

animals are also treated with PMDP (a new MDP derivative). We further show that this enhancement in activity is not due to increased production of interferon, but probably results from a PMDP-induced increase in nonspecific resistance to infection.

## 2. MATERIALS AND METHODS

### 2.1. *Animals*

Randomly bred Swiss mice weighing 14–15 g were used in all experiments. All the animals had access to food (pellet diet, Hindustan Lever, Bombay) and water ad libitum. Each experimental group consisted of an equal number of male and female animals.

### 2.2. *Virus*

SFV (Smithburn and Haddow strain) originally obtained from the American Type Culture Collection was maintained in our laboratory by intracerebral passage in mice. A 20% homogenate of the infected mouse brain in PBS containing 0.1% bovine serum albumin was lyophilized and stored at –20°C. The stock virus was titrated before use

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*Abbreviations:* SFV, Semliki Forest virus; VSV, vesicular stomatitis virus; PMDP, *N*-palmitoylmuramyl-L-alanyl-D-isoglutamine; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; PBS, phosphate-buffered saline (pH 7.2)

by subcutaneous inoculation in mice, and LD<sub>50</sub> was calculated using the formula of Reed and Muench [7]. VSV was obtained from the National Institute of Virology, Pune (India) and stored at -50°C. The virus was titrated in LM cells to calculate 50% tissue culture infective dose.

### 2.3. Mycoviral ds RNA

Mycoviral ds RNA was prepared from 9-day-old stationary cultures of the fungus *Aspergillus ochraceus* ATCC 28706, as described in [8].

### 2.4. Muramyl dipeptides

MDP was prepared as in [9]. PMDP was synthesized by conventional methods, and characterized by FAB/FD mass spectrometry (unpublished).

### 2.5. Animal experiments

Unless mentioned otherwise, ds RNA (0.6 mg/kg) and PMDP (in 0.5 ml PBS) were administered intraperitoneally to mice. The animals were challenged with SFV subcutaneously, and observed for a period of 20 days to record symptoms and mortality. The day of challenge is referred to as day 0 while other days are referred to as minus or plus (before and after the challenge, respectively) to indicate the days of treatment. The significance (*p* value) of the various treatment schedules was calculated by the test of proportions [10].

### 2.6. Interferon assay

Mice treated with ds RNA, PMDP or ds RNA plus PMDP were bled at various time intervals, and the serum separated and assayed for interferon. Interferon assay was carried out by the plaque reduction method on LM cell monolayers using VSV as the challenge virus. Reference mouse interferon procured from the National Institute of Health, USA (WHO international reference preparation, cat. no. G-002-904-511) was used as a standard for comparison, and to facilitate expression of interferon titre in IU/ml.

## 3. RESULTS AND DISCUSSION

Recovery from viral infections is a complex process, and depends mainly upon the interplay of host defence mechanisms including the interferon

and immune responses [11]. In appropriate cases interferon is produced in the blood stream within hours of virus inoculation or during viraemia and is distributed in target organs, conferring cellular resistance against further spread of virus [12] followed by antibody response which starts later during infection. The harmonious action of both interferon and immune responses may be necessary for the host to recover from the lethal infection [13]. We have observed that mice infected with SFV show paralytic symptoms from day +4 onwards and all succumb to infection by day +9. If the animals were treated with an interferon inducer, a mycoviral ds RNA, 1 day before or just prior to challenge, about 60–70% recover from the infection. The mice that do not recover despite the ds RNA treatment (30–40%) develop symptoms and ultimately die between day +9 and +20. These animals were found to have interferon levels

Table 1  
Effect of PMDP treatment on the anti SFV activity of ds RNA

| Expt | Treatment     | PDPMP dose (mg/kg) | N  | Protection (%) | <i>p</i> |
|------|---------------|--------------------|----|----------------|----------|
| I    | PBS           | —                  | 10 | 0              | —        |
|      | ds RNA        | —                  | 10 | 60             | —        |
|      | ds RNA + PMDP | 0.6                | 10 | 50             | —        |
|      | ds RNA + PMDP | 1.5                | 10 | 60             | —        |
|      | ds RNA + PMDP | 3.0                | 10 | 80             | —        |
|      | ds RNA + PMDP | 6.0                | 10 | 100            | <0.1     |
|      | ds RNA + PMDP | 15.0               | 10 | 100            | <0.1     |
|      | ds RNA + PMDP | 30.0               | 10 | 90             | —        |
| II   | PBS           | —                  | 30 | 0              | —        |
|      | ds RNA        | —                  | 30 | 53             | —        |
|      | ds RNA + PMDP | 6.0                | 30 | 90             | <0.01    |

ds RNA was administered on day -1 while PMDP was given on day 0. Animals were challenged with 100 LD<sub>50</sub> of virus, and observed for a period of 20 days to record occurrence of paralysis and death. *N*, number of mice

similar to those which survived the infection (unpublished), suggesting that the interferon response alone may not be sufficient for effective protection against SFV infection. It was therefore considered appropriate to examine the effect of muramyl dipeptides on the antiviral activity of ds RNA, as these glycopeptides are known to possess strong immunomodulatory activity [1,2].

Table 1 shows that the mice treated with ds RNA 24 h before challenge with 100 LD<sub>50</sub> of SFV were protected against infection to an extent of 55–60% only. This protection was considerably enhanced (90–100%) upon administering a single dose of PMDP to the ds RNA-treated animals on day 0. The optimal dose of PMDP required to obtain the maximum effect was about 6 mg/kg. A similar treatment with MDP (6 mg/kg) or with several other MDP derivatives (not shown) did not

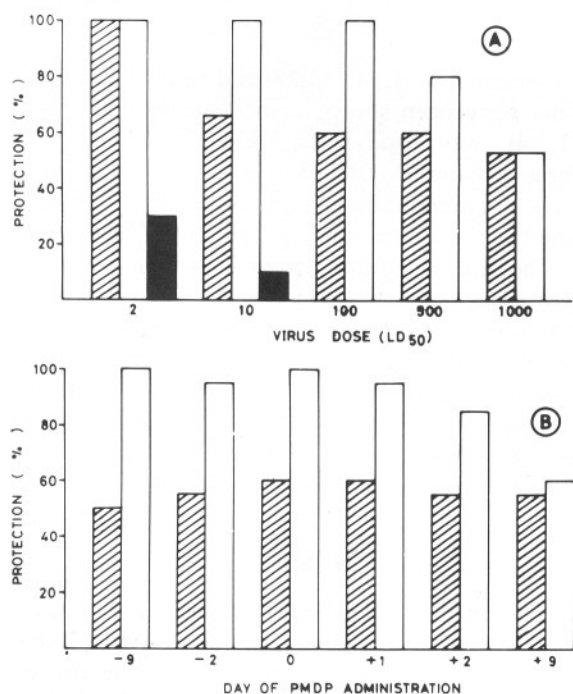


Fig.1. Effects of viral dose (A) [PMDP (6 mg/kg) was given on day 0] and the day of PMDP administration (B) (The doses of virus and PMDP were 100 LD<sub>50</sub> and 6 mg/kg, respectively. No animal in the control group survived up to day +20.) on SFV infection in ds RNA-treated mice. Bars: solid, control mice given 0.5 ml PBS only; shaded, ds RNA-treated mice; open, ds RNA plus PMDP-treated mice. Each group consisted of 15–20 animals. ds RNA was given on day -1.

significantly increase (70–80% protection) the antiviral activity of ds RNA.

Protection of the ds RNA plus PMDP-treated mice depended on the challenge dose of the virus (fig.1A). These animals were fully protected up to a viral dose of 100 LD<sub>50</sub>, but increasing the dose to  $\geq 500$  LD<sub>50</sub> led to a decrease in the extent of protection. Besides this, PMDP-induced enhancement in antiviral activity of ds RNA was also influenced by the day of PMDP administration (fig.1B). The animals that received the PMDP treatment be-

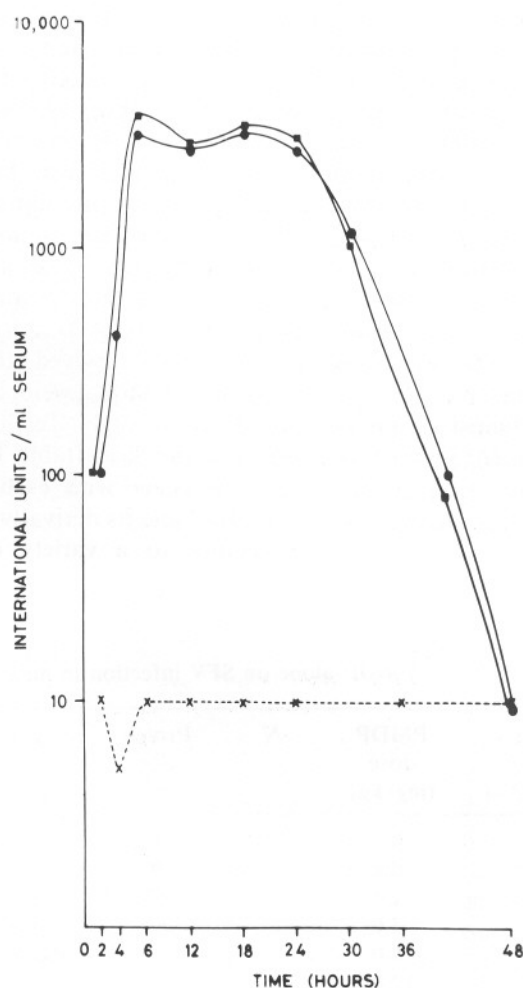


Fig.2. Interferon titres in serum of mice that were given ds RNA (■—■), PMDP (6 mg/kg: ×---×) or ds RNA plus PMDP (●—●). In the case of the combined treatment with ds RNA plus PMDP, PMDP (6 mg/kg) was given 4 h before ds RNA administration.

tween day -9 and +2 were effectively protected against SFV infection. However this treatment was not effective if given on day +9. No attempts were made to test the efficacy of the PMDP treatment beyond day +9 or -9.

To determine whether the PMDP-induced increase in antiviral activity of ds RNA is due to an increased production of interferon, we measured the interferon titres in serum of mice treated with ds RNA, PMDP or ds RNA plus PMDP. Fig.2 shows that PMDP alone did not induce interferon production, nor did it influence the kinetics of interferon synthesis in ds RNA-treated animals. This demonstrates that PMDP is neither an interferon inducer nor an activator of the interferon-inducing ability of ds RNA. It may, therefore, be inferred that the observed enhanced efficacy of the ds RNA plus PMDP treatment against SFV infection is not due to an increased production of interferon, but seems to result from a PMDP-induced stimulation of some specific and/or nonspecific immune response in the animals. That this indeed is the case was supported by our finding that mice treated with PMDP alone resist infection (table 2). Also, the PMDP-treated animals that survived the primary challenge with 2 LD<sub>50</sub> of SFV developed sufficient immunity to resist strongly further challenge with higher doses of the virus (table 3). These findings are quite consistent with earlier studies which showed that MDP and its derivatives enhance nonspecific resistance to a variety of

Table 2

Effect of PMDP alone on SFV infection in mice

| Virus dose (LD <sub>50</sub> ) | PMDP dose (mg/kg) | N  | Protection (%) | p     |
|--------------------------------|-------------------|----|----------------|-------|
| 2                              | 0                 | 15 | 30             | -     |
|                                | 0.6               | 15 | 30             | -     |
|                                | 1.5               | 15 | 53             | -     |
|                                | 3.0               | 15 | 80             | <0.01 |
|                                | 6.0               | 15 | 93             | <0.01 |
|                                | 12.0              | 15 | 73             | -     |
| 10                             | 0                 | 10 | 0              | -     |
|                                | 6.0               | 10 | 10             | -     |
|                                | 12.0              | 10 | 0              | -     |

PMDP was administered on day 0. N, number of mice

Table 3

Rechallenge of mice which recovered from primary challenge with SFV

| Animal group | Rechallenge dose (LD <sub>50</sub> ) | N  | Protection (%) | p     |
|--------------|--------------------------------------|----|----------------|-------|
| PBS-treated  | 10                                   | 11 | 18             | -     |
| PMDP-treated | 10                                   | 28 | 57             | <0.05 |
| Control      | 10                                   | 10 | 10             | -     |
| PBS-treated  | 100                                  | 9  | 0              | -     |
| PMDP-treated | 100                                  | 26 | 50             | <0.05 |
| Control      | 100                                  | 10 | 0              | -     |

PBS- and PMDP-treated animal groups were those surviving mice which were treated separately on day 0 with PBS and PMDP (6 mg/kg), respectively, at the time of primary challenge (2 LD<sub>50</sub>), while the control group consisted of normal mice which had never been infected. N, number of animals

pathogens [3-6]. As MDP and related glycopeptides have been shown to activate macrophages [1,2,4], we propose that possible activation of these cells by PMDP may increase the efficacy of ds RNA-induced interferon response by controlling the replication and/or spread of the virus.

The present study demonstrates that the antiviral activity of an interferon inducer is considerably enhanced by PMDP. Since several non-pyrogenic but highly potent MDP derivatives [2], including PMDP (unpublished), are now available, a combined treatment with muramyl dipeptide and an interferon inducer offers a new possibility for the effective control of viral infections.

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