CENTRAL PYROGENIC ACTIVITY OF MURAMYL DIPEPTIDE*

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Since the major discovery of Beeson (1), numerous reports have established that bacterial pyrogens induce the production of a circulating mediator that can act directly on the fever centers (2, 3). This serum factor, which seems to be identical to leukocyte pyrogen (LP),[1] elicits a monophasic fever of short latency and duration (3), probably by stimulating synthesis of derivatives of arachidonic acid in the anterior hypothalamic area (4, 5). However, some findings have suggested that, besides this common effector, bacterial products may also act directly on thermosensitive brain structures (5–7).

N-acetylmuramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP), a small molecular weight (<500) synthetic immunoadjuvant (8–10), has been shown to have a definite pyrogenic effect (11–13), and to elicit LP both in vitro (13) and in vivo (14). The fever response was completely abolished by indomethacin treatment, although the production of endogenous pyrogen was not inhibited.[2] The present study was designed to determine whether this compound could have a direct effect on the brain centers besides its indirect effect through the production of LP (13). In comparison, an adjuvant-inactive MDP-stereoisomer and MDP components were also administered by the intracisternal route.

Materials and Methods

Synthetic Compounds. MDP was MDP-Pasteur (Institut Pasteur Production, Paris). Its stereoisomer *N*-acetylmuramyl-D-alanyl-D-isoglutamine [MDP (D-D)] and the dipeptide (DP), L-alanyl-D-isoglutamine, were prepared by Lefrancier et al. (15) as previously described. The MDP sugar moiety *N*-acetylmuramic acid (AcMur) was obtained from the same batch used for synthesis. All the solutions were controlled by the Limulus amoebocyte lysate test, performed as previously reported (16). MDP and other synthetic compounds were always negative at 100 μg/ml.

Rabbit Pyrogen Assay. Male New Zealand white rabbits that weighed between 2 and 3 kg

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[1] *Abbreviations used in this paper:* AcMur, *N*-acetylmuramic acid; CNS, central nervous system; CSF, cerebrospinal fluid; DP, dipeptide; LP, leukocyte pyrogen; MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine; MDP(D-D), *N*-acetylmuramyl-D-alanyl-D-isoglutamine; PBS, phosphate-buffered saline; ΔT(°C), changes in temperature expressed as deviation from the baseline recorded at the time of injection.

[2] Parant, M., G. Riveau, F. Parant, C. Dinarello, S. Wolff, and L. Chedid. Effect of indomethacin on increased resistance to infection and on febrile responsee induced by muramyl dipeptide. Manuscript submitted for publication.

were used throughout the study. All glassware, needles, syringes and phosphate-buffered saline (PBS) were pyrogen free. Rabbits that did not have a stable temperature base line during at least the preceding 30 min were discarded. Rectal temperature was recorded every 15 min for 5 h after the injection with thermistor probes connected to a telethermometer (Ellab, Copenhagen). Changes in temperature are expressed as deviation from the base line recorded at the time of injection (ΔT[°C]).

Implantation of Cannula into the Cerebral Ventricle. A cannula was implanted in an aseptic operation into the right cerebral ventricle under pentobarbital anesthesia (25 mg/kg i.v.). The cannula used and the method of implantation were essentially the same as described by Feldberg in 1953 (17). Benzyl penicillin powder was sprinkled onto the wound, and 2,000 U was administered subcutaneously after the operation. Animals were used only 8 d later. Injections (20 μl/kg) were made through the rubber diaphragm of the cap, with controls receiving PBS only. In certain experiments, collection of cerebrospinal fluid (CSF) was performed by the same route 4 h after intracerebroventricular injection of MDP.

Passive Transfer of Blood. Heparinized blood from donors was collected aseptically from the carotid under pentobarbital anesthesia. Plasma was immediately separated by centrifugation in the cold at 2,000 *g* for 15 min and dialyzed overnight at 4°C with sterilized dialysis tubing against 200 vol of PBS to eliminate possible residual MDP. On the following day, plasma was prewarmed to 37°C before being slowly injected into the recipient rabbit by the intravenous route (10 ml/kg).

Results

Compared Pyrogenicity of MDP after Intravenous or Intracerebroventricular Administration. MDP was administered intravenously at 10, 30, 50, 100, and 300 μg/kg. A good dose-response curve was obtained, and the minimal pyrogenic dose of MDP that can induce a rise in temperature of 0.6°C corresponds to 25 μg/kg, as shown by the log dose-response line (Fig. 1). Typical fever patterns produced by a single intravenous injection of 30 or 300 μg of MDP are also depicted in Fig. 1. As is often seen with other pyrogens, a biphasic fever was produced with the higher dose, whereas the lower dose elicited only a monophasic response (Fig. 1).

Intracisternal injections of MDP were made at doses varying between 1 and 10^{-4} μg/kg with 10-fold dilutions. Again, a good dose-response curve was obtained with a tremendous increase of sensitivity because the minimal pyrogenic dose corresponds to 0.13 ng/kg (Fig. 2), and, therefore, was ~200,000-fold smaller than by the intravenous route. In contrast to Fig. 1, the fever pattern was monophasic at all doses. Moreover, at a higher dosage level such as 1 μg/kg (not reported in Fig. 2), temperature remained elevated for 24 h or more, whereas in all other cases it was reversed after 5 or 6 h. However, treatment with indomethacin, which has been shown to inhibit MDP-induced fever,[2] completely prevented the rise of temperature elicited by the intracerebroventricular injection of 1.5 ng/kg of MDP, which corresponded to 10 minimal pyrogenic doses.

Comparison of Endogenous Pyrogen Release in Plasma after Intravenous of Intracerebroventricular Administration of MDP (Fig. 3). Because a previous study has shown us that transferable circulating endogenous pyrogen can be demonstrated after an intravenous injection of MDP (14), similar experiments were performed after intracerebroventricular administration. All donor rabbits uniformly received 300 μg of MDP either intravenously or by intracerebroventricular injection, this dose represents ~10 minimal pyrogenic doses by the first route and 2×10^6 doses by the latter. Controls received the same volume of PBS either by intracerebroventricular or by intravenous route (not reported in Fig. 3). The temperature of all donors was recorded before

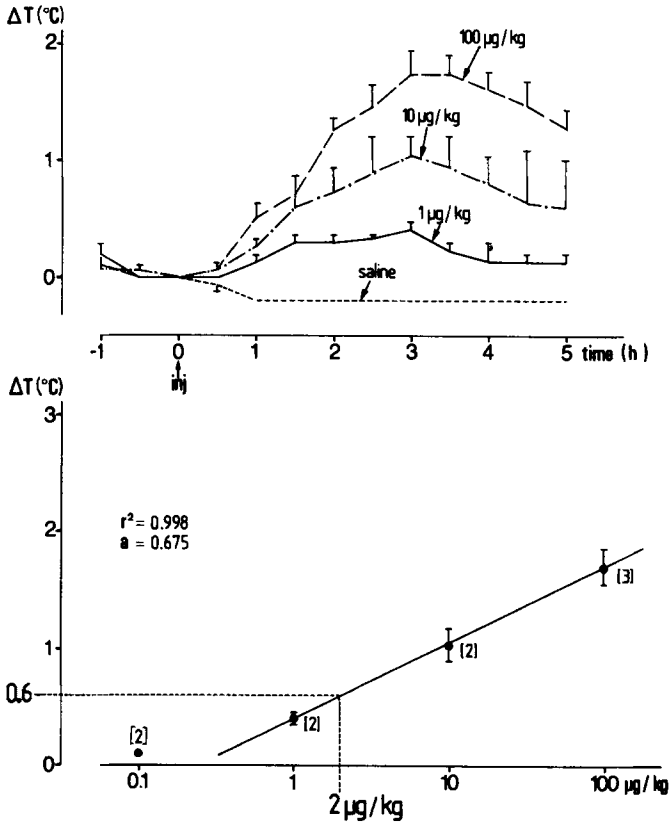

FIG. 1. Febrile response of the rabbit to intravenous (iv) administration of MDP. Results are given as mean ± SD; number of rabbits are given in parentheses. inj, injection; a, slope; r^2, coefficient of correlation.

bleeding. Blood was collected, in all cases, 2.5 h after intravenous administration, and either 2.5 or 5 h after intracerebroventricular injection. Plasma samples were dialyzed overnight and injected into untreated recipients on the following day. Fever responses were recorded during 3 h.

As usual, plasma collected from donors that had received MDP intravenously elicited a monophasic response with a peak at 1 h, typical of circulating endogenous pyrogen. However, all samples collected from donors that had received MDP by the intracerebroventricular route 2.5 or 5 h previously were unable to increase the recipients' temperature, although 300 µg of MDP administered by this route was repeatedly shown to produce hyperthermia during several days. Plasma of PBS-treated controls was also negative.

Absence of Endogenous Pyrogen in Cerebrospinal Fluid after Intracerebroventricular Administration of MDP. In experiments that will be published separately, samples of the pyrogenic supernate obtained from rabbit cells (10^7/ml) incubated with MDP were injected either by the intravenous or by the intracerebroventricular route. It was observed that 2 µl/kg induced a strong febrile response by intracerebroventricular route, whereas, by the intravenous route, 0.5 ml/kg of the same supernate was required to obtain a similar response. Therefore, fever can also be elicited after

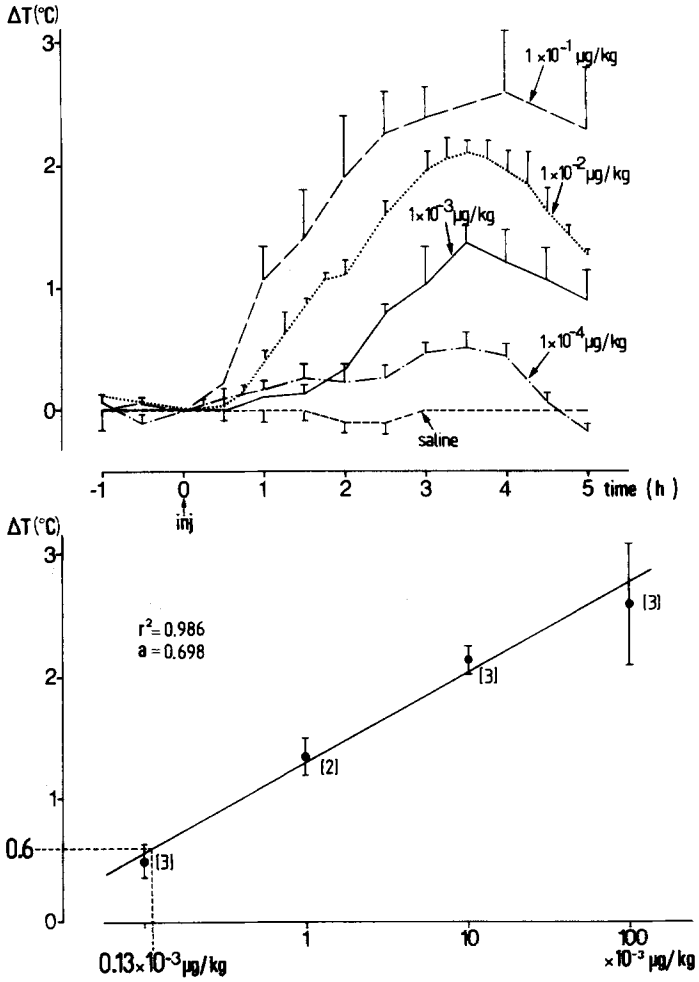

FIG. 2. Febrile response to intracerebroventricular (icv) administration of MDP. Results are given as mean ± SD; number of rabbits are given in parentheses, inj, injection; a, slope; r^2, coefficient of correlation.

intracisternal injection of MDP-induced endogenous pyrogen. Because circulating LP could not be detected in the plasma after intracerebroventricular injection of the glycopeptide, the following experiments were performed to find whether MDP could produce LP through the cells present in the cerebrospinal fluid.

Six rabbits received 0.5 µg/kg of MDP by the intracerebroventricular route and CSF was collected through the implanted cannulae 4 h later, at time of fever peak. Pooled CSF was dialyzed overnight against a large volume (1 liter) of PBS to eliminate both residual MDP and prostaglandins. On the following day, the presence of endogenous pyrogen was evaluated in three new recipients by an intracerebroventricular injection of 20 µl/kg of the dialyzed pool. No elevation of temperature was observed, although minute amounts of LP can be detected by this route.

Pyrogenicity of an Inactive MDP Stereoisomer and of MDP Components after Intracerebroventricular Injection. The adjuvant-inactive stereoisomer, MDP(D-D), which is not py-

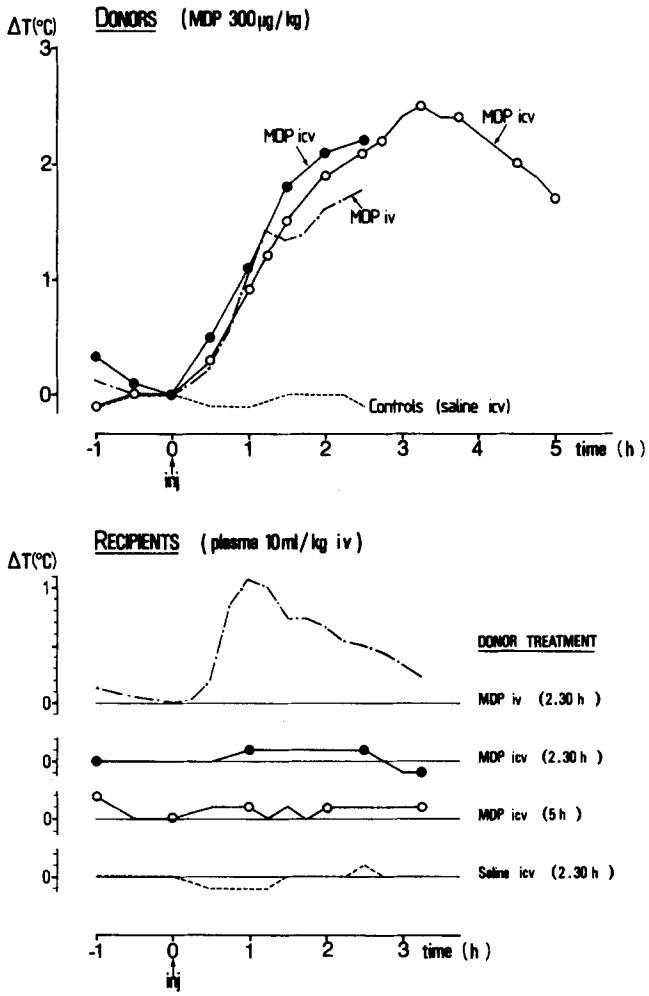

FIG. 3. Passive transfer experiments of plasma from rabbits made febrile by intravenous (iv) or intracerebroventricular (icv) injection of MDP. Results are given as mean ± SD for three rabbits per group, donors or recipients. inj, injection.

rogenic after intravenous administration of 10 mg/kg, was injected intraventricularly at doses varying between 100 and 0.1 µg/kg with 10-fold dilutions. A good dose-response curve was obtained with a monophasic fever pattern (Fig. 4), although the stereoisomer was ~20,000-fold less pyrogenic than MDP administered by the same route. Moreover, one cannot completely exclude some contamination by MDP during the preparation of its stereoisomer.

MDP contains a sugar moiety, AcMur, and a DP, L-alanyl-D-isoglutamine. These components were administered together or separately by the intracerebroventricular route. As shown in Fig. 5, even when high dosages (50 or 100 µg) of DP or AcMur were administered, separately or together, no fever was observed in contrast to the strong elevation induced by 0.01 µg/kg of MDP.

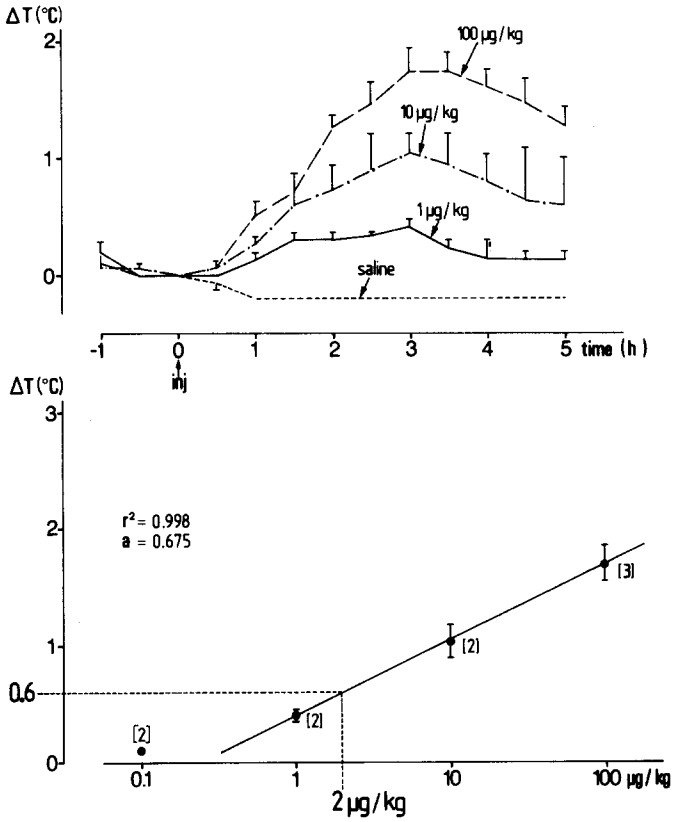

FIG. 4. Febrile response to intracerebroventricular administration of MDP(D-D). Results are given as mean ± SD; number of rabbits are given in parentheses. inj, injection; a, slope; r^2, coefficient of correlation.

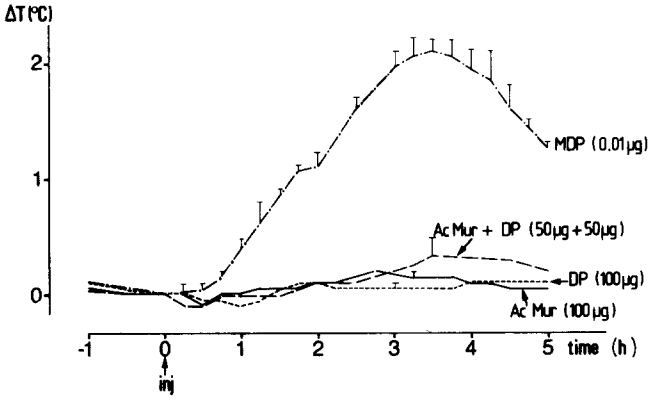

FIG. 5. Absence of pyrogenicity of MDP moieties administered by the intracerebroventricular route. Results are given as mean ± SD of three rabbits per group. inj, injection.

Discussion

The findings reported here demonstrate that the intracerebroventricular route potentiates tremendously (~200,000-fold) the pyrogenicity of MDP. Under such

conditions, a strong and lasting febrile response was elicited, although no circulating endogenous pyrogen could be detected in the plasma or in the CSF.

It has previously been shown that by the intravenous route, both amino acids are required to induce a febrile response to MDP because AcMur-L-alanine and AcMur-D-isoglutamine were inactive (18). The present study, performed by the much more sensitive intracerebral route, confirms that MDP represents the minimal requirement for inducing the febrile response ascribed to bacterial peptidoglycan (11, 12). Thus, neither the DP nor the sugar moiety injected together or separately at a dosage 10^6-fold greater than MDP, elicited any rise of rabbit temperature. It must be recalled that the MDP components, and the MDP stereoisomer used in this study as well, are devoid of immunostimulant properties (10, 16). Yet, after intracerebroventricular injection, MDP(D-D) induced a febrile response, but at dosage levels 10,000-fold higher than for MDP. All preparations were controlled by the Limulus amoebocyte lysate test to rule out the presence of endotoxic contamination. Absolute proof of an intrinsic pyrogenicity of the stereoisomer is lacking, however, because a minor amount of MDP cannot be totally excluded.

The extraordinary effectiveness of this small molecular weight glycopeptide in inducing fever after an intracerebroventricular injection raises again the question of the role of endogenous pyrogen in the mechanism of fever production (4). The requirement of LP release to produce fever can be bypassed because a febrile response can be directly elicited by intracerebroventricular administration of bacterial pyrogens such as gram-negative lipopolysaccharide (7), or streptococcal peptidoglycan (12, 19) and exotoxin (20). However, more recent studies have confirmed a previous evidence that lipopolysaccharide does not cross the blood-brain barrier (21). Endogenous pyrogen can also produce fever when injected by the intracerebroventricular route (22), but evidence for circulating LP entering the central nervous system (CNS) is also lacking (4). Such studies have not yet been performed with other pyrogens. Because of its small molecular weight, and although it is cleared very rapidly (23), the synthetic glycopeptide could more readily have access to the thermoregulating centers. Moreover, preliminary experiments have shown us that in rabbits made leukopenic by nitrogen mustard treatment, intravenous injection of MDP could produce fever in the absence of circulating plasma endogenous pyrogen. Such a dissociation was confirmed by intracisternal administration, which resulted in high fever without the production of endogenous pyrogen in the plasma or in the CSF. Similarly, Harvey and Milton (22) have previously found that central administration of endotoxin produces fever without activating the release of peripheral endogenous pyrogen. This does not exclude the possibility that centrally produced prostaglandins may be involved in the febrile response because they may enter the CNS through the cerebral circulation in amounts sufficient to produce fever (21). Moreover, responses to centrally administered pyrogens can be inhibited by antipyretic drugs, which block the prostaglandin system synthesis (4). The data reported here also show that indomethacin can abolish fever produced by intracisternal administration of MDP.

In summary, our present study strongly suggests that MDP can be pyrogenic by a direct effect on brain structures that control the body temperature. It is noteworthy to recall that even in the case of large molecular weight lipopolysaccharide, the possibility that toxin fragments could penetrate and act directly on CNS thermosensitive structures has never been completely ruled out.

Summary

Fever can be elicited in the rabbit by the intravenous administration of relatively large doses of a synthetic immunoadjuvant, *N*-acetylmuramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP). This response could be mediated by endogenous pyrogen because MDP has been shown to induce their production both in vivo and in vitro. The results reported here show that intracisternal injection of minute amounts of MDP could elevate fever without activating the release of endogenous pyrogen in the plasma or in the cerebrospinal fluid. Moreover, indomethacin inhibited hyperthermia produced by intracerebroventricular administration of MDP. Therefore, our findings argue in favor of a direct effect of the glycopeptide on the thermoregulatory centers besides its indirect effect through the production of leukocytic pyrogen. This molecule apparently represents the minimal requirement for the pyrogenicity of bacterial peptidoglycan because administration, even by the intracerebral route, of a mixture of muramic acid and of its dipeptide moiety did not elicit fever.

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