Specific absorption with monoclonal antibodies to muramyl dipeptide of the pyrogenic and somnogenic activities of rabbit monokine

(endogenous pyrogen/slow-wave sleep/immunoadsorbent)

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ABSTRACT It is well established that muramyl dipeptide (MDP) can induce fever and enhance slow-wave sleep. Recently, crude or purified supernatants of activated macrophages containing endogenous pyrogen (EP) were also shown to enhance slow-wave sleep. These similarities and the recent finding that a mammalian factor that enhances slow-wave sleep is a muramyl peptide triggered us to study the possibility of the presence of this bacterial structure in the EP molecule. In the present study, EP was produced by stimulation of rabbit peritoneal cells with a nonpyrogenic, nonsomnogenic analog of MDP. The EP-containing supernatant lost its pyrogenicity and somnogenicity after passage over an immunoadsorbent column of monoclonal anti-MDP but not of another monoclonal antibody of different specificity. High percentage of the EP was recovered by elution of the anti-MDP columns with HCl/glycine buffer. Results suggest that bacterial muramyl peptides may be incorporated by mammalian cells into substances that act in picomole quantities to mediate immunological and physiological processes. In addition, the technique may be useful to extract interleukin 1 for structural studies.

A sleep-promoting factor in cerebrospinal fluid from sleep-deprived goats was described by Pappenheimer and colleagues >15 yr ago (1, 2). More recently, a muramyl peptide having sleep-promoting properties, similar to the cerebrospinal fluid factor, was isolated from brains of sleep-deprived rabbits and from human urine (3). The active factor(s) from each of these sources specifically enhanced slow-wave sleep (SWS) in rats, rabbits, and cats. Synthetic muramyl dipeptide (MDP) and several of its adjuvant-active analogs were also shown to enhance SWS (4, 5). The somnogenic muramyl peptides, natural or synthetic, were also pyrogenic and it was shown that a pyrogenic purified interleukin 1 (IL-1) is strongly somnogenic (6). A close relationship between endogenous pyrogen (EP), sometimes designated by IL-1, and muramyl peptides was revealed by monoclonal antibodies raised against MDP: these antibodies bound to sleep-promoting factor purified from urine or brain, and they also inhibited the IL-1 enhancement of phytohemagglutinin-induced thymocyte proliferation (7).

We now describe preparation of an immunoadsorbent column (IAC) of monoclonal anti-MDP antibody that specifically binds the pyrogenic and somnogenic activities contained in supernatants of activated rabbit macrophages. The active factor, presumably IL-1, can be eluted in high yield by HCl/glycine buffer. These experiments provide evidence that endogenous pyrogen (IL-1) contains an MDP-like structure or binds nonspecifically to MDP. In either case, the IAC itself may be used to prepare relatively large quantities of highly purified IL-1 for structural studies.

MATERIALS AND METHODS

MDP and Derivatives. MDP was MDP-Pasteur (Institut Pasteur Production, Paris). The following MDP analogs were prepared by P. Lefrancier, who has reported their synthesis elsewhere (8): AcMur-L-Ala-d-Gln α-n-butyl ester [MDP-(Gln)-OMe] or murabutide, and AcMur-L-Ala-d-Gln α-methyl ester [MDP(Gln)-OMe] or murametide. Murametide stimulates macrophages to secrete EP in vitro (9), but unlike MDP it has no pyrogenic or somnogenic actions (5, 9). Murabutide reacts with antibody to MDP but does not stimulate EP production and has no pyrogenic or somnogenic effects (9, 10).

Monoclonal Antibodies. The preparation and characterization of monoclonal anti-MDP antibodies has been described elsewhere (11). In this study, two such monoclonal antibodies were used—namely, 2-4-5 (IgG3) and M-52-11 (IgG2a). By using the previously described ELISA for detection of specificity, both monoclonal antibodies were found to bind to MDP and were not capable of recognizing either the sugar (N-acetylmuramic acid) or the dipeptide (L-Ala-d-isoGln) separately. Monoclonal anti-dinitrophenyl (DNP) antibody (namely, U-7-27), prepared as described (12), was used as a control and was kindly provided by Z. Eshhar (Weizmann Institute of Sciences, Israel). In some experiments, immunoglobulin fractions (50% ammonium sulfate precipitation) of ascitic fluid were used in the preparation of IAC. In other experiments, an IgG fraction of ascitic immunoglobulin isolated on Sephacryl S-200 column (Pharmacia) was used in the preparation of the IAC.

Preparation of IAC. This was carried out according to the manufacturer's instructions. Briefly, cyanogen bromide-activated Sepharose 4B (Pharmacia) was washed on sintered glass filters (G3) with 1 mM hydrochloric acid. The gel was immediately mixed with monoclonal antibody (either as ascitic immunoglobulin or as IgG fraction) dissolved in 0.1 M sodium bicarbonate buffer (pH 8.3) containing 0.5 M sodium chloride (referred to as coupling buffer) at a ratio of 5 mg of protein per ml of gel. The coupling step was carried out overnight at 4°C with gentle end-over-end mixing. The amount of protein coupled on the gel was in all cases >90% of the total protein added, as determined spectrophotometrically at 280 nm. After washing uncoupled protein from the resin, the remaining active groups on the gel were blocked by mixing the

Abbreviations: DNP, dinitrophenyl; EP, endogenous pyrogen; IAC, immunoadsorbent columns; i.c.v., intracerebroventricular; IL-1, interleukin 1; MDP, muramyl dipeptide for N-acetylMuramyl-L-Ala

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gel with 0.2 M glycine (pH 8.0) for 2 hr at room temperature. The gel was further washed 5 times alternately with coupling buffer followed by acetate buffer (0.1 M; pH 4.0) to remove adsorbed protein. It was then poured into a 10-ml syringe or a C 10/10 Pharmacia column and washed extensively with pyrogen-free phosphate-buffered saline (pH 7.4). Nonspecific protein binding was further blocked by passing 20 mg of bovine serum albumin over each column, followed by washing with buffer, and eluting with 0.1 M glycine/HCl, pH 2.8. The columns were then equilibrated with phosphate-buffered saline (pH 7.4) before applying the active peritoneal exudate cells (PEC) supernatants. The flow rate was 0.5 ml/min for packing, 0.2 ml/min for sample passage, and 0.5 ml/min for elution.

Preparation of Cell Supernatants. Adherent cells from rabbit peritoneal exudate (107 per ml) were cultured under conditions reported elsewhere (13). Briefly, they were incubated for 18 hr with 100 µg of murametide per ml or other MDP analogs in serum-free medium and then dialyzed overnight against buffer to remove low-molecular-weight glycopeptides.

Rabbit Pyrogen Assay. New Zealand White male rabbits (2.5–3.5 kg) were used throughout the study. All glassware, needles, and syringes, and phosphate-buffered saline were pyrogen-free. Injections were made via the marginal ear vein (i.v.) or through the cannula (20 µl/kg) inserted into the lateral ventricle (intracerebroventricular, i.c.v.) according to procedures previously described (14). Febrile response to MDP- or murametide-induced EP are reported as the maximum change in body temperature. Highest temperatures were usually at 45 min after i.v. injection and 3.5 hr after i.c.v. administration. Changes in temperature are expressed as deviation from the baseline recorded at the time of injection (ΔT°C ± SD). The minimal pyrogenic dose represents the amount capable of inducing an increase of 0.6°C in rabbit temperature.

Measurement of Rabbit SWS. Aliquots (10–50 µl) of supernatants to be tested were diluted to 0.3 ml with pyrogen-free artificial cerebrospinal fluid and were infused i.c.v. into male New Zealand rabbits at a rate of 7 µl/min. Infusions were carried out between 08:00 and 10:00 hr and were followed by 6 hr of electroencephalographic recording as described (4).

RESULTS

Specific Absorption of EP from Murametide-Activated PEC on IAC of Monoclonal Anti-MDP Antibody. The supernatants of unstimulated cells or of the murabutide-activated cells did not elicit fever after i.v. injection (0.5 ml/kg) to rabbits (Table 1).

Table 1. Specific absorption of EP on IAC of monoclonal anti-MDP antibodies

<table>
<thead>
<tr>
<th>Supernatant of macrophages activated with (100 µg/ml)</th>
<th>Supernatant absorbed on IAC of</th>
<th>ΔT</th>
<th>°C ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.08 ± 0.07 (6)</td>
<td></td>
</tr>
<tr>
<td>Murabutide</td>
<td></td>
<td>0.13 ± 0.09 (6)</td>
<td></td>
</tr>
<tr>
<td>MDP</td>
<td></td>
<td>0.72 ± 0.07 (6)</td>
<td></td>
</tr>
<tr>
<td>Murametide</td>
<td></td>
<td>0.60 ± 0.06 (7)</td>
<td></td>
</tr>
<tr>
<td>Anti-MDP (M-52-11)</td>
<td></td>
<td>0.04 ± 0.08 (5)</td>
<td></td>
</tr>
<tr>
<td>Anti-MDP (2-4-5)</td>
<td></td>
<td>0.2 ± 0.08 (3)</td>
<td></td>
</tr>
<tr>
<td>Anti-MDP (2-4-5) blocked with murabutide</td>
<td></td>
<td>0.67 ± 0.05 (3)</td>
<td></td>
</tr>
<tr>
<td>Anti-DNP (U-7-27)</td>
<td></td>
<td>0.64 ± 0.05 (5)</td>
<td></td>
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</tbody>
</table>

Number of rabbits is given in parentheses. Control was phosphate-buffered saline.
*Change in temperature after i.v. injection of supernatant at 0.5 ml/kg (not concentrated).

As expected, supernatants of both MDP- and murametide-treated cells contained pyrogenic activity. A 4-fold concentrated supernatant (5 ml) of murametide-activated macrophages was passed twice over a 10-ml IAC of ascitic Ig of (i) U-7-27 (monoclonal anti-DNP); (ii) M-52-11 (monoclonal anti-MDP); (iii) 2-4-5 (monoclonal anti-MDP); or (iv) 2-4-5 (monoclonal anti-MDP) that had been blocked with 20 mg of murabutide prior to application of the active supernatant. The supernatant from each column was collected in a final vol of 20 ml (4× void volume). The results of testing the supernatant before and after passage over the columns are presented in Table 2 as maximum change in temperature after i.v. injection (ΔT°C ± SD). The pyrogenic activity was retained on the anti-MDP columns (M-52-11 and 2-4-5). In contrast, no apparent loss of pyrogenic activity was observed after passage of supernatant over anti-DNP column (U-7-27). Moreover, after being blocked by murabutide the anti-MDP IAC no longer retained pyrogenicity, indicating that this retention was specific.

Elution of EP from Monoclonal Anti-MDP IAC. In the following experiment, another murametide-treated PEC supernatant was passed under the same conditions on IAC of IgG fraction from ascitic immunoglobulin of either U-7-27 (anti-DNP) or of M-52-11 (anti-MDP). After collecting the first eluate as described above in a final vol of 20 ml, each column was washed with 10 ml of buffer then eluted with 20 ml of 0.1 M glycine/HCl, pH 2.8. The pH of the eluate was neutralized to 7.0 with 1 M Tris-HCl and the eluate was concentrated 4-fold by using an immersible ultrafiltration unit (Millipore), to a vol of 5 ml in preparation for the biological assay.

In this case, to evaluate pyrogenic activity in different eluates, the i.c.v. route was used. This route is much more sensitive, because the minimal pyrogenic dose is ~0.2 µl/kg instead of 0.5 µl/kg after i.v. administration. Changes in temperature after i.c.v. administration of the eluates of each column, as well as of the supernatant before passage over the columns, are reported in Table 2. The minimal pyrogenic dose of the murametide-treated PEC supernatant before passage through the columns was <0.3 µl/kg. In contrast, even at 2.5 µl/kg the eluate of the anti-DNP column was not pyrogenic. However, pyrogenic activity was present in the eluate of the anti-MDP column. In this case, a dose of 0.3 µl/kg was sufficient to induce febrile responses, and 0.6 µl/kg gave the same increase in temperature as 0.3 µl/kg of the control supernatant, suggesting that ~50% of the activity has been recovered.

Table 2. Elution of EP from monoclonal anti-MDP IAC

<table>
<thead>
<tr>
<th>Supernatant of murametide-activated macrophages</th>
<th>Volume injected, µl/kg</th>
<th>ΔT</th>
<th>°C ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.07</td>
<td>0.20 ± 0.10 (3)</td>
<td></td>
<td></td>
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<tr>
<td>0.15</td>
<td>0.43 ± 0.05 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.85 ± 0.18 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate of anti-DNP IAC (U-7-27)</td>
<td>0.60</td>
<td>0.20 ± 0.01 (2)</td>
<td></td>
</tr>
<tr>
<td>1.25</td>
<td>0.32 ± 0.15 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>0.37 ± 0.25 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate of anti-MDP IAC (M-52-11)</td>
<td>0.15</td>
<td>0.20 ± 0.10 (2)</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.60 ± 0.08 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.60</td>
<td>0.83 ± 0.17 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.50</td>
<td>1.13 ± 0.05 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.50 (heated)</td>
<td>0.13 ± 0.13 (4)</td>
<td></td>
<td></td>
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</table>

Number of rabbits is given in parentheses.
*Change in temperature after i.c.v. injection of supernatant (not concentrated).
Although supernatants were carefully dialyzed, to ensure that the pyrogenic activity of the eluate was unrelated to residual amounts of muramyl peptides or to contamination by exogenous pyrogens (endotoxins), the active eluate was heated at 56°C for 30 min. After heating, even at 2.5 μg/kg the eluate failed to produce hyperthermia. This treatment destroys endogenous pyrogen but has no effect on endotoxins or on pyrogenic synthetic muramyl peptide.

Absorption and Elution of SWS Activity of Supernatants from Murametide-Activated PEC on IAC of Monoclonal Anti-MDP Antibody.

Pyrogenic supernatants of activated macrophages as well as affinity-purified EP were shown to enhance SWS (6). We assayed, therefore, for pyrogenic and pyrogenic activities of IAC supernatants before and after passage through IACs. Furthermore, eluates of anti-DNP (U-7-27) or anti-MDP (M-52-11) IACs were checked for these activities. Results presented in Table 3 (mean of two separate experiments with different supernatants and different IACs) show that the pyrogenic supernatants were also pyrogenic. After passage over an anti-DNP IAC, neither of these activities was lost. In contrast, both activities were retained on the IAC of monoclonal anti-MDP antibody. Moreover, both somnogenicity and pyrogenicity were demonstrated in the eluates of the anti-MDP but not in the eluates of the anti-DNP IAC (Table 3).

**DISCUSSION**

The study reported here was carried out in view of two purposes: (i) a possible procedure to extract and purify IL-1, and (ii) pursuing previous experiments that suggested an MDP-like structure in mammalian mediators. Early attempts to purify IL-1 have been unsatisfactory; at best, very low yields were obtained (15–17). The possibility of using IACs has recently been explored by Mizel et al. (18), who used polyclonal antibodies raised in goats against purified supernatants containing IL-1. In the present investigation, we have observed high yields of IL-1 by using an IAC prepared with a monoclonal antibody to MDP. The specificity of absorption was demonstrated by lack of retention of EP over an IAC of the same monoclonal antibody blocked with another nonpyrogenic MDP derivative (murabutide). However, our results do not exclude the possibility of nonspecific absorption of murametide to the generated IL-1 in cultures. The monokine could then be absorbed on the IAC of monoclonal anti-MDP by virtue of the attached murametide and not because of specific recognition of IL-1. In any case, it appears likely, therefore, that this technique can be developed to obtain highly purified IL-1 in quantities required for structural studies. Whether an MDP-like structure of the same or of different configuration also exists in supernatants of other IL-1-producing cells activated by MDP or by other immunostimulants still remains to be elucidated.

Table 3. Somnogenic and pyrogenic activities of PEC supernatants absorbed on and eluted from IACs of monoclonal anti-MDP antibody.

<table>
<thead>
<tr>
<th>Sample tested</th>
<th>% SWS</th>
<th>Δt, °C ± SD</th>
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</thead>
<tbody>
<tr>
<td>Supernatant of murametide-activated macrophages</td>
<td>43 ± 1</td>
<td>54 ± 2 ± (5)</td>
</tr>
<tr>
<td>Supernatant absorbed on anti-DNP (U-7-27) IAC</td>
<td>39 ± 1</td>
<td>50 ± 3 (5)</td>
</tr>
<tr>
<td>Supernatant absorbed on anti-MDP (M-52-11) IAC</td>
<td>42 ± 1</td>
<td>42 ± 1 (6)</td>
</tr>
<tr>
<td>Eluate of anti-DNP (U-7-27) IAC</td>
<td>40 ± 1</td>
<td>46 ± 3 (8)</td>
</tr>
<tr>
<td>Eluate of anti-MDP (M-52-11) IAC</td>
<td>42 ± 1</td>
<td>50 ± 1 (8)</td>
</tr>
</tbody>
</table>

Exp, experimental. Number of rabbits is given in parentheses. For % SWS data, the same rabbits were used before and after injection.

% SWS during a 6 hr post-injection period of 20 μl of sample.

Maximun change in temperature after i.v. injection of sample at 0.5 ml/kg.

Significant difference from control, P < 0.05. Each rabbit was its own control; paired statistics were used.
the host requires but cannot synthesize. Recognition of anti-MDP monoclonal antibodies of mediators capable of enhancing SWS as well as of eliciting fever strongly supports this view.

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