Somnogenic Activity of Pseudomurein in Rabbits

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Pseudomurein is a major cell wall component of some archaeabacteria that chemically differs from but morphologically, functionally, and structurally resembles eubacterial peptidoglycan. Eubacterial cell wall components, e.g., peptidoglycan, induce changes in sleep and body temperature. We now report that intravenous injections of rabbits with a suspension of pseudomurein from Methanobacterium thermoautotrophicum also induce similar central nervous system effects.

Pseudomurein is a peptidoglycan structure found in the cell walls of members of the order Methanobacteriales (12). This substance and eubacterial peptidoglycan serve to prevent osmotic disruption, and they are morphologically similar. Both types of peptidoglycan consist of glycan strands cross-linked by short peptides, and they seem to have similar three-dimensional architectures (16–18). However, the chemical composition of pseudomurein differs considerably from that of eubacterial peptidoglycan: the glycan of pseudomurein contains N-acetyl-talosaminuronic acid instead of muramic acid, the sugars are connected by β-1,3-glycosidic linkages, and the peptide contains a remarkably high number of non-α-bonded amino acids but no D amino acids (8, 12).

Eubacterial infections elicit a series of transient changes collectively known as the acute-phase response, within hours of inoculation. These responses include central nervous system (CNS) effects, such as changes in temperature and sleep. Dead bacteria (22) and eubacterial peptidoglycan (6), as well as other eubacterial cell wall products, such as endotoxin (14), induce similar CNS responses in mammals. For example, increased slow-wave sleep (SWS) and body temperature are observed after intravenous (i.v.) injection of rabbits with cell walls of Staphylococcus aureus or Neisseria gonorrhoeae (6). In contrast, little is known about biological effects of pseudomurein, although methanogenic bacteria occur in the rumens and intestinal tracts of a variety of animals (19, 20). For example, Methanosphaera cuniculi, a pseudomurein-containing species, has been detected in the intestinal tracts of rabbits (2). However, there are no known interactions between these organisms and the host (12), and, to our knowledge, no CNS effects of pseudomurein have been reported previously. We now report that pseudomurein, given systemically, alters sleep and brain temperature ($T_{br}$) in rabbits.

Lyophilized Methanobacterium thermoautotrophicum was a gift from O. Kandler and H. König. Pseudomurein was isolated as described previously for staphylococcal cell walls (5, 15). In preparation for i.v. injection into rabbits, the lyophilized pseudomurein was suspended in appropriate volumes of pyrogen-free saline (PFS). In some cases, it was suspended in PFS containing 1 mg of polymyxin B sulfate (Sigma, St. Louis, Mo.) per ml and preincubated for 1 h at 37°C prior to the injection. This was done to inactivate possible endotoxin contamination, which is common in reagents. In a separate control experiment, polybead polystyrene microspheres (0.98-μm diameter; 2.5% solids = 5 × 1010 spheres per ml; Polysciences, Wissington, Pa.) were diluted to 5 × 1010 per ml in PFS before i.v. injection.

Amino acid analysis of the pseudomurein sample was performed by Jerome M. Seyer (Veterans Administration Medical Center, Memphis, Tenn.). As anticipated (12), only three amino acids were detected: alanine (2.08 nmol/mg of pseudomurein), glutamic acid (1.84 nmol/mg), and lysine (1.79 nmol/mg). No other amino acids were found at a sensitivity that would have detected <50 pmol/mg. Amino sugar content was not determined. The Limulus amoebocyte lysate (LAL) assay (sensitivity, 0.25 endotoxin unit [EU/ml] and control standard endotoxin [CSE] (E. coli 0113; Associates of Cape Cod, Woods Hole, Mass.) were used to test samples for endotoxin contamination.

Rabbits (pasterella- and coccidium-free) were kept on a 12-h light–12-h dark cycle (0600 to 1800 h) at 21 ± 2°C. Food and water was available ad libitum. The rabbits were habituated overnight to the experimental cages before each recording session. One milliliter of sample suspension or vehicle was injected i.v. between 0830 and 0900 h. Electroencephalograph (EEG), $T_{br}$, and movement were continuously recorded for 6 h following the injection. Colonic temperatures ($T_{col}$) were determined before the injection and after 6 h. The EEG was band-pass filtered (0.5 to 3.5 Hz; 8; 4 to 8 Hz, 8). The signal from each filter was rectified, averaged for 1-min periods with EEG analyzers (Buxco model 24/36DL), and digitally recorded every minute. These values were used to calculate the mean $\delta$-wave voltage; they reflect both length of time spent in SWS and the amplitudes of individual $\delta$ waves. They are used as an independent means to validate SWS values determined by visual scoring. The polygraph records were visually scored in 12-s epochs for periods of SWS, rapid eye movement sleep (REMS), and wakefulness as previously described (7, 13). Experimental and control recordings were obtained from the same rabbits on different days. Control injections were PFS; controls for pseudomurein-plus-polymyxin samples received a solution of 1 mg of polymyxin B per ml of PFS, which was preincubated at 37°C for 1 h prior to injection. Average hourly values were used to evaluate effects across the 6-h recording period by means of the Wilcoxon matched-pairs signed-ranks test.

The injection of 1 to 2 mg of pseudomurein per rabbit did not result in significant effects on SWS, REMS, or EEG $\delta$-wave voltages (Table 1), nor did it affect $T_{br}$ or $T_{col}$ (data not shown). The injection of 4 mg of pseudomurein per rabbit increased SWS significantly over the 6-h recording period.

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investigate whether the observed pseudomurein effects on sleep and $T_{br}$ were due to the particulate or to the chemical nature of the sample, polystyrene microspheres were injected via the same route as and at a concentration comparable to that of pseudomurein ($5 \times 10^5$ sacculi were contained in 4.5 mg of a cell wall preparation of *Staphylococcus aureus*, that was prepared in the same way as the pseudomurein [6]). Polystyrene microspheres 1 $\mu$m in diameter are phagocytized by macrophages in vitro (9), and microspheres 3 $\mu$m in diameter injected i.v. into dogs are found in phagocytizing cells (10). The injection of $5 \times 10^6$ microspheres in 1 ml of PFS had no effects on sleep (Table 1) or on $T_{co}$ or $T_{br}$ (data not shown). The failure of these polystyrene microspheres to elicit sleep and temperature effects excludes the possibility that any suspension of particles with sizes similar to those of bacteria is capable of eliciting the observed effects by activation of macrophages. A second control experiment tested the possibility that endotoxin contamination might have contributed to the observed sleep effects. The LAL test was used to estimate and polymyxin B sulfate was used to inactivate any possible endotoxin contamination. A suspension of 1 mg of pseudomurein per ml of PFS contained a LAL reactivity of between 1.25 and 12.5 ng of CSE per ml. This LAL activity of 1 mg of pseudomurein per ml was inactivated by 1 mg of polymyxin B sulfate per ml. A concentration of 0.01 mg of polymyxin B sulfate per ml was sufficient to inactivate 10 ng of CSE per ml in the LAL assay. Because a much higher dose of polymyxin B was needed to inactivate the pseudomurein LAL activity than to inactivate CSE activity, this suggests that pseudomurein itself had LAL activity. It has been shown that the LAL assay is not specific for endotoxin and that eubacterial peptidoglycan contains LAL reactivity 3 to 5 orders of magnitude less potent than that of endotoxin (1, 23). Thus, the effects of pseudomurein in the LAL test might be considered a further aspect of biological activity of this substance, possibly because of the structural similarity to eubacterial peptidoglycan.

When pseudomurein was preincubated with 1 mg of polymyxin B sulfate per ml prior to injection (four animals with 2 mg and four animals with 4.5 mg of pseudomurein), SWS and $\delta$-wave voltages were significantly increased and a slight but nonsignificant enhancement of REMS was observed (Table 1). A transient increase in $T_{br}$ occurred and had magnitude, shape, and timing equal to those of the increase observed after injection of pseudomurein that was not pretreated with polymyxin B (data not shown).

These observations of effects of pseudomurein on sleep and $T_{br}$ in rabbits are to our knowledge the first observations of CNS effects of this substance. Although only limited

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### TABLE 1. Effects of i.v. injection of pseudomurein or microspheres on rabbits

<table>
<thead>
<tr>
<th>Pseudomurein dose (mg)</th>
<th>n*</th>
<th>Means ± SEM*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>SWS (% time)</td>
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<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1–2</td>
<td>4</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>47 ± 2</td>
</tr>
<tr>
<td>2–4.5</td>
<td>8</td>
<td>46 ± 1</td>
</tr>
</tbody>
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* n, number of rabbits.

* **, P < 0.01; ***, P < 0.005 (Wilcoxon matched-pairs signed-ranks test).

* Pseudomurein pretreated with polymyxin B.

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FIG. 1. Effects of i.v. injection of 4 mg of pseudomurein in 1 ml of PFS (●) or vehicle (1 ml of PFS into same rabbits on different days) (○) on rabbit SWS (A), REMS (B), $\delta$-wave amplitudes (C), and $T_{br}$ (D). (A through C) Values are hourly averages for the indicated hours (6-h averages are shown in Table 1). (D) Values are differences from control recording ($\Delta T_{br}$). Datum points are means ± standard error of the mean (n = 4). SWS and EEG $\delta$-wave voltages were increased during postinjection hours 1 through 4. REMS was not affected. $T_{br}$ was transiently increased, with a peak 1.5 h postinjection.
information on the biological effects of pseudomurein is available, it was demonstrated earlier that in a rat arthritis model, intra-articular injection of high doses of pseudomurein-poly saccharide fragments from *methanobacterium formicicum* caused an acute inflammation (21). Also, pseudomurein was shown to be antigenic in animals and humans (4).

The pathways by which pseudomurein exerts its somnogenic and pyrogenic effects are unknown. Pseudomurein is resistant to lysozyme (8), the key enzyme for the degradation of eubacterial peptidoglycan in macrophages. Also, the accumulation of unusual $\epsilon$ and $\gamma$ bonds in pseudomurein may protect it against attack by proteases (8). However, some enzymatic degradation processes for pseudomurein have been described recently: an extracellular enzyme produced by a streptomy cete from cow manure has the capability to lyse a pseudomurein-containing methanogen (3). Also, a peptidase that hydrolyzes the $\epsilon$-Ala-Lys bond of pseudomurein was purified from *methanobacterium wolfei* (11). Whether such processes are involved in the biological response of pseudomurein remains undetermined.

This study was supported in part by grants from the Deutsche Forschungsgemeinschaft, the National Institutes of Health (NS-25378, NS-27250, MH-47103), the Office of Naval Research (contract no. N00014-90-J-1069), and the U.S. Army Medical Research and Development Command (contract no. DAMD-17-86-C-6194). We thank Donna Maxwell, Gail Richmond, and Roy Broady for their assistance.

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