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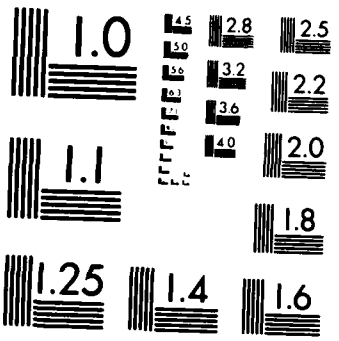

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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Previously, we had identified muramyl peptides (MPs) as components of mammalian tissues and shown that they are somnogenic. Current research is focused on three areas: 1) the quantitative occurrence of MPs in mammalian tissues; 2) how is sleep linked to the immune system; and 3) do other immune response modifiers alter sleep? In the 1986-87 year, we have: a) developed methods to isolate diaminopimelic acid (dap, a component of MPs from mammalian tissue) and quantify dap in those tissues; b) shown that mammalian macrophages release somnogenic low molecular weight substances during the digestion of bacteria; c) described binding sites for MPs on macrophages; d) shown that muramyl dipeptide induces, via a central nervous system mechanism, alterations in one measure of the host defense response, plasma Cu; e) shown that bacterially infected animals do, in fact, sleep more than normal; f) demonstrated that endotoxin and its lipid A component are somnogenic; g) shown that several lymphokines, i.e., tumor necrosis factor, interferon-alpha, and recombinant interleukin-1, are also somnogenic. We conclude that alterations in sleep are an additional facet of the host defense response to infectious agents.

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Introduction and Background

A long-term interest of our laboratory has been to isolate and characterize substances that accumulate in brain during wakefulness and induce sleep. The first such somnogenic substance we isolated from rabbit brains and human urine is a muramyl tetrapeptide (NAG-1,6 anhydro NAM-ala-glu-dap-ala) (18, 19, 20, 21). Muramyl peptides (MPs) are also the monomeric building blocks of bacterial cell wall peptidoglycan and have been extensively studied as immune-response modifiers. Thus, our work has led to the investigation of relationships between the immune response (broadly defined) and sleep. For example, it was known that MPs have the capacity to induce interleukin-1 (IL1) synthesis and release and that IL1 was a glia cell product; thus, we subsequently showed that it is also somnogenic (4, 22). Subsequently, others showed that IL1 cerebrospinal fluid levels varied in phase with sleep-wake cycles. Further, since there are no known synthetic pathways in mammals for some of the components of MPs, e.g., muramic acid and diaminopimelic acid (dap), the question has been raised whether MPs play a role in normal physiology, perhaps analogous to that of vitamins, i.e., substances required by but not synthesized by the host.

Our current research ~~is thus~~ focused on three general areas: A) MPs - are they present in mammalian tissue, how do they get there, and what are their target sites; B) how is sleep linked to the immune response; and C) do other immune response modifiers alter sleep? Our published work (1986-1987) concerning each of these areas is summarized below.

PROGRESS - 1986-1987

A. Muramyl Peptides (MPs)

1. Levels of muramic acid and dap in mammalian tissues. Muramic acid (23), dap (24) and MPs (20) are present in normal mammalian tissues, and MPs have multiple biological activities in mammals, although the quantitative levels of MPs in mammalian tissue remain unknown. It was desirable, therefore, to develop a method to estimate MP levels by measuring dap levels in mammalian tissues. Our first experiments have focused on developing chromatographic techniques to isolate and measure the amount of dap found in free and in hydrolyzable linkage in human urine (an inexpensive, readily available tissue). Samples were collected from male humans and immediately frozen. At the start of the cleaning procedure, small amounts of radioactive dap were added to samples so that we could determine recovery of dap. Cleaning steps included precipitation of proteins, solid phase extraction of nonpolar substances, anion and cation exchange chromatography, and reversed-phase chromatography. Purified samples of dap were derivatized to form W-HFB-i-butyl ester derivatives. They were separated using gas chromatography isothermally on a medium polar capillary column and detected with an electron capture detector. Detection limits of this method are in the low pmol range. In samples tested to date, we found between 200 and 700 pmol of free dap per ml of urine and a daily output of free dap in the range of 1 pmol via urine (16). This method is now being adapted to measure the amount of dap in hydrolyzable linkage and also to measure dap and muramic acid as well in other mammalian tissues.

2. Macrophage processing of bacteria; centrally active substances are produced. Macrophages phagocytize and partially digest bacteria; thus, a

likely source for MPs in mammals could be the digestion products from cell walls. Indeed, there is one report suggesting that MPs are released by macrophages during the digestion of bacteria (25), but it was unknown if those MPs were biologically active in mammals. We have therefore begun to examine whether murine macrophages release biologically active substances of low molecular weight during the digestion of bacteria. Bone marrow-derived murine macrophages were fed viable Staphylococcus aureus that were ^{14}C-marked in their cell walls. Supernates of the macrophages were collected after 3, 12, 48, and 96 h of digestion, pooled, and freeze-dried. The samples were then processed by gel filtration, and fractions containing ^{14}C in the molecular weight ranges between 5,000 D and the salt volume were pooled and dried. After the samples had been dissolved in artificial cerebrospinal fluid, they were heated to 60°C for 30 min to eliminate possible biological activity of lymphokines. Small amounts (50 μl) of these samples, which contained about 500 pmol of dap in hydrolyzable linkage, were injected into a lateral cerebral ventricle of male rabbits; and electroencephalograms (EEG), brain temperature, and movement were recorded for 6 h during daytime.

The infusion of the macrophage supernate resulted in enhanced slow-wave sleep (SWS) and brain temperature and in decreased rapid eye movement (REM) sleep (26). The time courses of these effects were similar to those observed after MP administration, but not to those elicited by lymphokines and prostaglandins. The processing of bacterial walls into products of low molecular weight by macrophages is a daily occurrence, as well as an early event in the initiation and amplification of the immune response. That such products are also active in the central nervous system (CNS) suggests a role for MPs in sleep regulation, as well as an early involvement of the CNS in the immune response.

3. Binding sites for MPs on macrophages (5). Although it is well known that muramyl dipeptide, NAM-L-ala-D-isogln (MDP), has multiple effects on macrophages and is somnogenic (21), the cellular basis for these effects is poorly understood. Therefore, we sought to determine whether macrophages possessed specific binding sites for MPs. Two radiolabeled [^{125}I]MP derivatives of high specific activity were prepared: a tripeptide with an iodinated C-terminal tyrosine methyl ester (Ligand I), and a muramyl tripeptide with a C-terminal lysine derivatized with Bolton-Hunter reagent (Ligand II). These were used to characterize binding of MPs to monolayers of murine macrophages. Saturable high-affinity binding to resident, caseinate-elicited, and Listeria-activated peritoneal cells was observed with both radioligands. Binding affinities varied with the state of activation of the macrophages, and K_D values ranged from 48 ± 33 pM (for resident macrophages, Ligand I) to 1,020 ± 90 pM (for activated macrophages, Ligand II). Specific binding sites were also found on a macrophage-derived cell line. The ability of several unlabeled MPs to compete with Ligands I and II for their binding sites was tested. Competition was stereospecific and correlated with known biological activities of these compounds (i.e., immunoadjuvanticity, pyrogenicity, and somnogenicity). The sites identified here for Ligands I and II may mediate some of the effects that MPs have previously been demonstrated to have on macrophages.

B. Links Between the Immune Response and Sleep

1. Effects of MDP on sleep, body temperature and plasma copper (Cu) after intracerebroventricular (i.c.v.) administration (13). Many MPs, such as

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MDP, are immune response modifiers, pyrogenic, and somnogenic. The purposes of this study were to measure the somnogenic effects of MDP in conjunction with a biochemical measure of the host defense response, plasma Cu, and to determine if plasma Cu levels, like sleep, are regulated by a CNS process. MDP administered into a lateral cerebral ventricle induced a dose-dependent rise in plasma Cu at 28 h postinfusion. This was associated with dose-dependent fevers, increases in SWS, and reductions in REM sleep during the first 6 h after injection of MDP. Intravenous (i.v.) administration of the same amount of MDP did not affect any of these variables. We conclude that the syndrome induced by centrally administered MDP includes activation of the host defense response with respect to a rise in plasma Cu, in addition to fever and enhanced sleep.

2. Effects of bacterial infections on SWS in rabbits (27). Increased body temperature and altered immune system activity are well-known features of infectious disease. Several compounds associated with infectious processes, including MPs and IL1, are pyrogens, immunomodulators, and somnogens. Subjective reports of increased "sleepiness" often accompany states of illness; however, the effects of infectious disease on sleep have not been evaluated systematically. To examine the effects of an infectious disease on sleep, EEG was monitored in 14 adult male New Zealand White rabbits during a 24-h control period and for 48 h following i.v. inoculation with 10^7 to 10^8 colony-forming units of Staphylococcus aureus. Despite variability in the time course and the magnitude of responses, every animal tested showed enhanced SWS following inoculation. The time spent in SWS increased by about 50% during a 6-16 h period postinoculation (PI) and then returned to control levels. The enhancement of SWS was associated with increases in both the amplitude of EEG slow waves (from 6-8 h PI) and the duration of individual bouts of sleep (6-16 h PI). After 18 h PI, both the amplitude of EEG slow waves and SWS bout duration dropped below control levels, eventually returning to baseline by 48 h PI. The inoculation also produced several changes that typically are associated with infectious disease. Rectal temperatures increased by 1-2°C within 6 h PI and remained elevated for 48 h. The febrile effects of the inoculation could thus be dissociated temporally from the somnogenic effects. Hematologic analysis revealed a severe lymphopenia in all animals and a marked neutrophilia in most animals tested. The lymphopenia generally persisted for 48 h PI. The neutrophil response was more variable in terms of both magnitude and duration. Those animals that failed to develop a strong neutrophil response tended to show a shorter duration of enhanced SWS and a more severe clinical progression than did animals with marked neutrophilia. Postmortem blood cultures were positive in 10 of the 14 animals tested.

These data thus demonstrate that inoculation of rabbits with Staphylococcus aureus results not only in the febrile and hematologic mainifestations of infectious disease, but also in a pronounced increase in SWS. The time courses of these effects differed substantially, suggesting that they result from separate mechanisms. We speculate that changes in SWS represent an adaptive response to infectious disease.

C. Other Immune Response Modifiers: Effects On Sleep

1. Enhancement of SWS by endotoxin and lipid A (8). Some MPs derived from bacterial peptidoglycan enhance SWS. The purpose of this study was to test whether another cell wall component, lipopolysaccharide (LPS), and its

lipid A moiety also have an effect on sleep. When injected i.v., both LPS and lipid A enhanced the duration of SWS, increased EEG δ-wave amplitudes, suppressed REM sleep, and induced biphasic fevers. The effects of i.v. administered lipid A and LPS on SWS were present primarily during the first 3 h post-injection. I.V. lipid A administration enhanced SWS, did not suppress REM, and induced a monophasic fever; the SWS effect had a 3-h latency, whereas temperature started to rise during the second hour. Regardless of the route of administration, within the dose range used, sleep was normal by the following criteria: sleep was episodic, animals could be easily aroused, and brain temperature, although elevated to febrile levels, continued to fluctuate during sleep-state transitions indistinguishably from control conditions. We conclude that LPS and lipid A are capable of modulating sleep.

2. Interferon α-2 enhances rabbit SWS (12). Interferon α-2 (IFN) is a leukocyte product with several biological properties, including antiviral activity, pyrogenicity, and enhancement of immune functions. An additional facet of IFN activity is its ability to enhance SWS without greatly altering other aspects of sleep. I.V. or i.c.v. injections of human IFN into rabbits induced enhancement of SWS, EEG slow-wave (0.5-4 Hz) activity, and brain temperatures. IFN induced slight reductions in REM sleep. Animal behavior, brain temperature changes that occur during the transition from one arousal state to another, and the cyclic nature of states of vigilance remained undisturbed after IFN treatment. The sleep-promoting activity of IFN may be related to feelings of lassitude and sleepiness that often accompany viral disease and interferon therapy.

3. Recombinant tumor necrosis factor (TNF) and IL1 enhance SWS (14). The cytokines IL1 and IFN are immune response modifiers that are also pyrogenic and somnogenic. TNF (cachectin) is another pyrogenic monocyte product whose production can be elicited by somnogenic agents such as endotoxin. Human recombinant TNF (rTNF), therefore, was assayed for somnogenic activity. I.V. or i.c.v. injections of rTNF enhanced SWS and EEG slow-wave (0.5-4.0 Hz) activity. Recombinant TNF also suppressed REM sleep and induced biphasic fevers whether given by i.v. or i.c.v. injection. Responses to rTNF were compared with those elicited by human recombinant β-IL1 (rIL1). Sleep responses elicited by rIL1 were similar to those previously reported for native IL1 and to those elicited by rTNF. However, unlike rTNF, rIL1 induced monophasic fevers. Animal behavior and brain temperature changes that occur during the transition from one arousal state to another remained undisturbed after either rTNF or rIL1 treatment. The fact that TNF and IL1 as well as other immunoactive substances, e.g., IFN, MPs, and endotoxin, enhance SWS suggests that SWS is linked to the immune response. We conclude that TNF, in addition to IL1 and IFN, is an endogenous somnogen.

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