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Rapid Elimination of a Synthetic Adjuvant Peptide from the Circulation after Systemic Administration and Absence of Detectable Natural Muramyl Peptides in Normal Serum at Current Analytical Limits

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Although it is clear that muramyl peptides are involved in sleep associated with bacterial infection, their role in normal physiological sleep is less certain. It has been speculated that "natural" muramyl peptides, derived from degraded gut flora, may pass into the bloodstream, where they play a role in normal sleep (M. Karnovsky, Fed. Proc. 45:2556–2560, 1986). Muramic acid serves as a chemical marker for muramyl peptides, since it is not synthesized by mammals. After injection of synthetic muramyl dipeptide in rabbits, muramic acid was readily detected (after release by acid hydrolysis) in the circulation; however, levels rapidly decreased. This was an important positive control in assessing circulating levels of natural muramyl peptides. Muramic acid was not found in normal serum (detection limit, approximately 500 pmol/ml), demonstrating the absence of appreciable amounts of circulating natural muramyl peptides. At this time we are unable to provide supportive evidence for Karnovsky's hypothesis.

Peptidoglycan polymers, oligomers, and monomers all have potent biological effects. It has been recognized for many years that group A streptococcal peptidoglycan-polysaccharide polymers are capable of causing chronic inflammatory diseases of joints and other tissues (6). A major factor in the induction of perpetuating effects in vivo is the ability of peptidoglycan-polysaccharide polymers to persist in depots in the reticuloendothelial system and elsewhere. Peptidoglycan-polysaccharide complexes remain in host tissues, since they are resistant to degradation by mammalian enzymes and avoid rapid excretion through the kidney (7, 12, 25). The extremely large size of these molecules is a major factor in their ability to cause chronic inflammation (3, 9).

Recently there has been a focus on the properties of small cell wall monomers (muramyl peptides) that cause a number of short-lived effects in vivo and in vitro. The most studied of these molecules has been muramyl dipeptide (adjuvant peptide; *N*-acetyl-muramyl-L-alanine-D-isoglutamine [MDP]), the minimal active subunit of bacterial peptidoglycan (2). However, other muramyl peptides that contain 1,6-anhydro-muramyl residues have been reported to have greater potency (15). Acute effects of muramyl peptides include breakdown of the blood-aqueous humor barrier (17), arthritis (26), cytoxicity (5), and slow-wave sleep (15). That cell wall monomers can cause sleep has excited much interest, since it explains one major side effect of infection—listlessness. More controversial has been a suggestion that muramyl peptides play a role in the sleep process of healthy people (16).

In support of this last suggestion has been the observation that normal tissues, including the brain, appear to contain trace amounts of muramic acid, presumably as a component of muramyl peptides (23). There is currently no evidence for biosynthesis of muramic acid by mammalian tissues; thus these muramyl peptides would have to be of exogenous origin (16).

Within a few hours of administration, radiolabeled MDP and peptidoglycan monomers are rapidly eliminated in the urine (1, 21, 24). There is also a mammalian enzyme (*N*-acetyl-muramyl-L-alanine amidase) that inactivates muramyl peptides by hydrolyzing the lactylamide bond between *N*-acetylmuramic acid and L-alanine, the first amino acid of the peptide side chain (14, 18, 20). Thus, if biologically active muramyl peptides are present in host tissues, there must be continuous replenishment from some bacterial source. It has been stated that the source for the putative muramyl peptides might be the large bacterial depots in the gut, which on degradation would generate muramyl peptides that could be absorbed into the circulation. Although this is an attractive hypothesis, there have been no studies on the levels of "natural" muramyl peptides in the circulation, and there is currently no evidence that these substances do, indeed, penetrate from the gut (16).

In work presented here our goal was to determine whether muramyl peptides could be detected in the normal circulation. Muramic acid served as a chemical marker for the presence of muramyl peptides, since it is not found in nature other than in peptidoglycan (11, 13). As a positive control the circulating levels of muramic acid, after systemic administration of synthetic MDP, were determined. Gas chromatography-mass spectrometry (GC-MS) was utilized, combining the high-resolution separating power of fused silica capillary gas chromatography with the selectivity of mass spectrometric detection.

Female New Zealand White rabbits weighing approximately 2 kg were injected with a total of 2 mg of MDP (Research Plus, Bayonne, N.J.) in phosphate-buffered saline (PBS) or with PBS alone through an ear vein; 30 min or 4 h later the animals were given a lethal dose of pentobarbital-

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ketamine, and blood was removed through an ear artery or by cardiac puncture. The blood was allowed to clot at 4°C, and serum was stored at −70°C until analysis.

Each serum sample (15 ml) was centrifuged in a Centriprep 10 unit (Amicon, Danvers, Mass.) through a filter with a molecular weight cutoff of 10,000. The serum filtrates were collected, lyophilized, and reconstituted in one-fourth of their original volume. Then 0.5 ml of fourfold-concentrated serum (i.e., equivalent to an original volume of 2 ml) was mixed with 0.5 ml of 4 N sulfuric acid and hydrolyzed at 100°C to release muramic acid from MDP and putative muramyl peptides. After cooling, methylglucamine was added as an internal standard. The mixture was neutralized with *N*,*N*-dioctylmethylamine (Fluka Chemical Corp., Haupage, N.Y.). The water phase was passed through a C-18 column to remove hydrophobic contaminants (J. T. Baker, Philippsburg, N.J.) and then reduced with sodium borohydride to destroy the anomeric center so that only one peak was produced for each sugar on GC-MS analysis. Acetic acid-methanol (1:200, vol/vol) was added, and the sample was dried repeatedly to remove borate, which otherwise inhibits the subsequent acetylation reaction. The samples were acylated with acetic anhydride (Alltech Associates, Deerfield, Ill.) at 100°C overnight to make muramic acid and other sugars volatile during subsequent GC-MS analysis. The samples were then extracted with acid and alkali to remove polar contaminants. Samples were analyzed in the electron ionization mode with a model 5890 gas chromatograph interfaced to a model 5970 mass spectrometer (Hewlett-Packard, Palo Alto, Calif.) containing an SP-2330 or SP-2380 fused silica capillary column (10).

GC-MS has been previously used to assess muramic acid levels in animal tissues after systemic administration of high-molecular-weight cell wall components (11–13) and in human body fluids after natural infection (4). However, GC-MS has not been used to measure the levels of circulating muramyl peptides in mammalian body fluids or tissues. The levels of muramic acid in serum were expected to be considerably lower than those in our previous work.

MDP is a small molecule with a molecular weight of 492; other muramyl peptides would be somewhat higher in molecular weight (generally around 1,000). Disposable Centriprep 10 units, which contain filters that eliminate molecules larger than 10,000 daltons, were used for the first time in our work. They provided a simple and effective clean-up procedure. The serum filtrate was completely colorless, indicating removal of hemoglobin and other proteins. Hydrolyzed serum (without prior cleanup), as expected, contained large amounts of glucosamine, whereas sera that had been passed through Centriprep columns contained virtually no glucosamine, indicating removal of glycoproteins. Presumably other large molecules, such as RNA and DNA, are also largely eliminated at this stage. Thus we were able to analyze relatively large volumes of serum, which significantly increased the sensitivity of analysis.

Our primary goal was to determine whether muramic acid, which serves as a natural chemical marker for MDP, could be detected in the circulation of mammals. As a positive control, 10 serum samples were analyzed in duplicate that were derived from blood sampled from rabbits 30 min after injection of MDP. In each case muramic acid was readily detected. A typical chromatogram is shown in Fig. 1A. The mean ± standard deviation levels of muramic acid were 651 ± 212 ng/ml of serum. Table 1 shows levels of MDP in individual animals. Four sera from animals injected with MDP 4 h previously did not contain detectable muramic

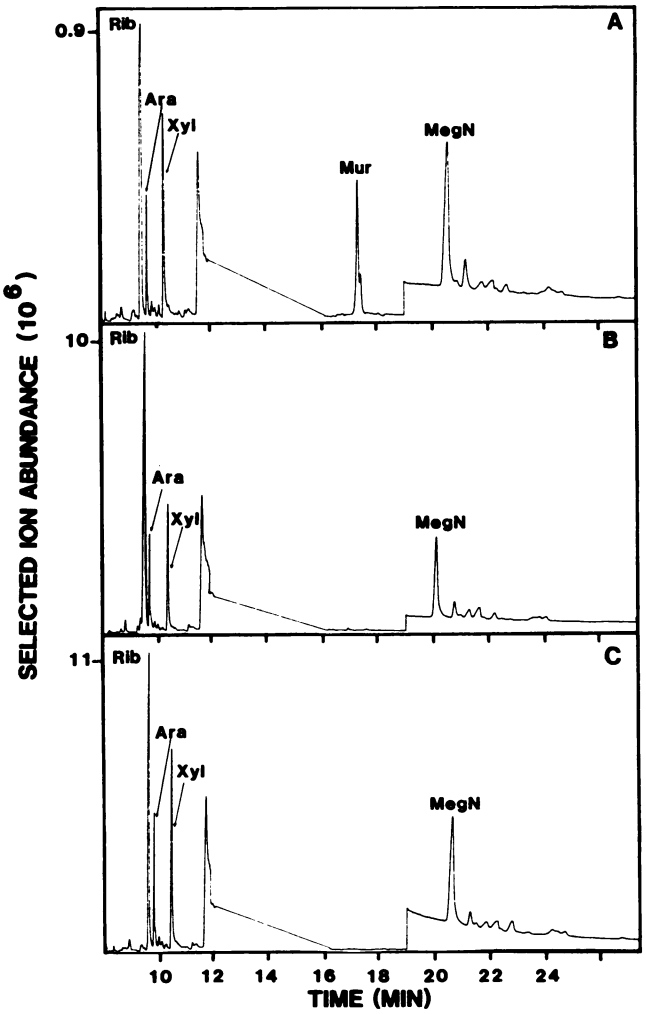

FIG. 1. Chromatograms of serum from rabbits injected (A) 30 min previously with MDP in PBS, (B) 4 h previously with MDP in PBS or (C) with PBS alone. Peaks: Rib, ribitol; Ara, arabinitol; and Xyl, xylitol (natural sugars found in serum); Mur, muramicitol; MegN, methylglucamine (internal standard). The mass spectrometer was turned off in the middle portion of the chromatogram to avoid overloading the ion source, since a huge peak eluted in the glucose region.

acid. In agreement with other data (22), all sera analyzed contained ribitol, arabinitol, and xylitol, which served as natural controls for the analysis. For quantitation, methylglucamine was added as an internal standard. A typical chromatogram showing the appearance of serum 4 h after injection of MDP is shown in Fig. 1B. The rapid elimination rate of MDP demonstrated in this study is consistent with previous work in which radiolabeled material was injected into mice (21).

A total of 16 normal serum samples were also analyzed in duplicate to determine muramic acid levels. Five samples were obtained by bleeding an ear artery, and the remainder were obtained by cardiac puncture. In no instance was muramic acid detected. Baselines were flat in the muramic acid region of chromatograms (Fig. 1C). It should be stressed that in every case methylglucamine (the added

TABLE 1. Levels of muramic acid in serum 30 min after intravenous injection of MDP

Serum no.	Concn of muramic acid (ng/ml)	Blood sampling route
1	532	Ear artery
2	582	Ear artery
3	625	Ear artery
4	770	Cardiac puncture
5	744	Cardiac puncture
6	691	Cardiac puncture
7	362	Cardiac puncture
8	672	Cardiac puncture
9	950	Cardiac puncture
10	635	Cardiac puncture

internal standard) and ribitol, arabinitol, and xylitol (normal components of serum) were reliably detected.

To assess the sensitivity of our approach, aliquots of a serum pool were spiked with 980, 490, 245, or 0 ng of MDP per ml (equivalent to 500, 250, 125, or 0 ng of muramic acid per ml, respectively) and processed through the entire analytical procedure. Each pool was analyzed in duplicate. As expected, the muramic acid peak increased in size with increasing MDP. Even in the samples spiked with 125 ng/ml, muramic acid was detectable; however, this appeared to be the current detection limit. Chromatograms of the spiked and unspiked serum pools are shown in Fig. 2C and D.

The mammalian host is constantly exposed to bacteria. There is extensive bacterial colonization of all external surfaces, with the largest amounts of bacteria present in the gut. Low-molecular-weight bacterial degradation products might constantly be generated from the normal flora, absorbed in the gut, and passed into the circulation (16). It is possible that bacterial remnants might also persist in normal host tissues after processing of bacteria during infection. There have been few studies of the levels of bacterial remnants in normal mammalian tissues (11, 12, 23).

Muramic acid (3-*O*-lactyl glucosamine) is an amino sugar that is a component of the backbone of bacterial peptidoglycan. There is no evidence for synthesis of muramic acid by mammalian enzyme systems. After systemic injection of high-molecular-weight streptococcal cell wall complexes, muramic acid is readily detected (after release from peptidoglycan-polysaccharide polymers by hydrolysis) in depots in various tissues and persists for extensive periods of time. In these studies, muramic acid was not found in normal mammalian tissues. However, small muramyl peptides would not have been detectable, since samples were first dialyzed to remove low-molecular-weight substances (11, 12).

In a qualitative study of muramic acid levels, it has been reported that a rough estimate of the level in liver of muramyl peptides is higher than 100 pmol/g (wet weight) of tissue. In this work muramic acid was isolated by thin-layer chromatography. The fluorescamine derivative of muramic acid was identified by periodate oxidation (indicating the presence of a sugar or other substance containing a diol) and alkaline release of lactic acid (which is characteristic but not specific for muramic acid). Since these identification procedures are not unique for muramic acid, the exact nature of the partially isolated substance remains to be confirmed by more selective approaches (16, 23).

In the present study muramic acid was not detectable in 16 normal serum samples, each analyzed in duplicate. The detection limit was less than 500 pmol/ml. It is an attractive hypothesis that solubilized bacterial remnants may be found in healthy animals after uptake from the gut of degraded bacterial flora. Unfortunately, we were unable to provide evidence to support this notion.

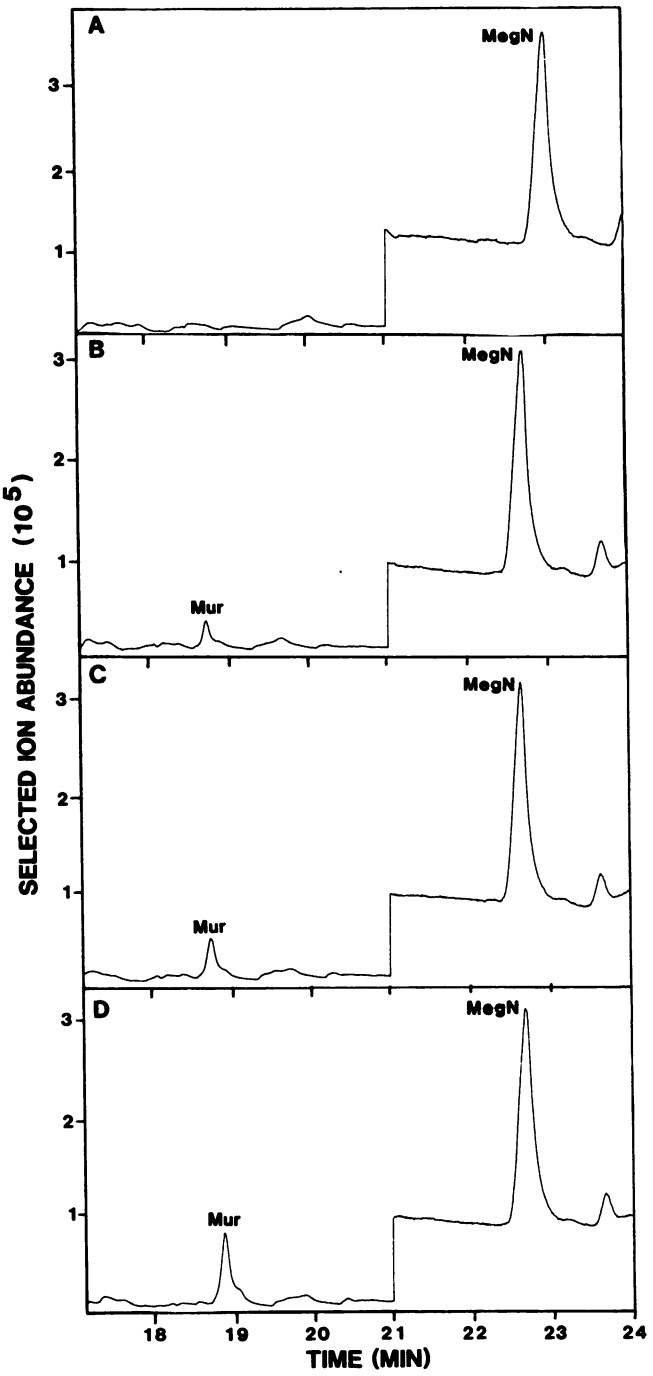

FIG. 2. Chromatograms of pooled serum (A) without MDP or (B through D) spiked with 125, 250, and 500 ng of muramic acid per ml, respectively. See Fig. 1 for identification of peaks.

After systemic adminstration of MDP, muramic acid was readily detected in sera after release by hydrolysis, derivatization, and GC-MS analysis. At 30 min after injection of

MDP, levels of muramic acid were around 651 ng/ml (2.6 nmol/ml). This served as an important positive control demonstrating our ability to detect muramyl peptides in vivo. MDP was rapidly eliminated from the circulation and was not detectable at 4 h after injection (at least fivefold-lower levels).

Some biological effects of MDP can be elicited with extremely low dosages. For example, MDP at 25 μg/kg enhances both slow-wave sleep and rapid eye movement sleep. In contrast, a higher dose of MDP (500 μg/kg) induces pronounced inhibition of rapid eye movement sleep (19). In the present work the quantity of MDP injected into rabbits corresponds to a dose that would inhibit rapid eye movement sleep. Doses of MDP that correspond to somnogenic effects would be present for short periods after injection in body fluids at levels below current detection limits.

In conclusion, it is possible that our failure to detect muramic acid in normal serum may be due to its presence at levels lower than present analytical limits. It is also entirely possible that others detected a substance or mixture of substances other than muramic acid in normal tissues (23). These two possible interpretations can only be resolved by the development of a more sensitive procedure that retains the selectivity and quantitative nature of the current state-of-the art in muramic acid analysis. There is a dearth of information on the biochemical processing and handling of bacterial peptidoglycan in vivo. Further studies on the levels of low- and high-molecular weight bacterial debris in vivo could prove highly important in our understanding of a variety of acute and chronic phenomena associated with bacterial infections (8).

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