Detection of Muramic Acid in a Carbohydrate Fraction of Human Spleen

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Received 6 October 1994/Returned for modification 12 December 1994/Accepted 29 January 1995

In previous studies, we showed that peptidoglycan polysaccharides from anaerobic bacteria normally present in the human gut induced severe chronic joint inflammation in rats. Our hypothesis is that peptidoglycan from the gut flora is involved in perpetuation of idiopathic inflammation. However, in the literature, the presence of peptidoglycan or subunits like muramyl peptides in blood or tissues is still a matter of debate. We were able to stain red pulp macrophages in all six available human spleens by immunohistochemical techniques using a monoclonal antibody against gut flora-derived antigens. Therefore, these human spleens were extracted, and after removal of most of the protein, the carbohydrate fraction was investigated for the presence of muramic acid, an amino sugar characteristic for peptidoglycan. Using three different methods for detection of muramic acid, we found a mean of 3.3 μmol of muramic acid with high-pressure liquid chromatography, 1.9 μmol with a colorimetric method for detection of lactate, and 0.8 μmol with an enzymatic method for detection of D-lactate per spleen (D-lactate is a specific group of the muramic acid molecule). It is concluded that peptidoglycan is present in human spleen not as small muramyl peptides as were previously searched for by other investigators but as larger macromolecules probably stored in spleen macrophages.

Muramic acid (Mur) is one of the two amino sugars in the glycan backbone of peptidoglycan (murein). It is mostly present in an acetylated form, i.e., N-acetylmuramic acid. Short peptides are linked to the lactyl groups of this molecule, forming a large complex macromolecule which surrounds the cell as a basket around the cell membrane (29). In this study, Mur was used as a chemical marker for peptidoglycan polymers. Sen and Karnovsky (20) showed the qualitative detection of muramic acid in normal mammalian tissues. Fox and Fox (5) were not able to detect Mur in normal serum, which demonstrates the absence of appreciable amounts of circulating natural muramyl peptides. Our group is interested in the presence of peptidoglycan in tissues, as we believe that such intestinal flora derived carbohydrates are important for physiological and immunological functions in the host. Johannsen et al. (11, 12) showed that muramyl peptides have a variety of biological actions in mammals, including the ability to enhance sleep and body temperature. In the last 15 years, many authors have described biological activities of peptidoglycan in vivo and in vitro, all indicating that peptidoglycan can influence cells like lymphocytes and macrophages which are involved in inflammation. Lichtman et al. (15) showed that livers of rats injected intraperitoneally with peptidoglycan were able to reactivate arthritis after transplantation into a second rat with preinjured joints. They postulate that peptidoglycan present in the liver is redistributed to other tissues, including the injured joints, where it causes reactivation of arthritis. From the studies done by Lichtman et al., it seemed obvious to look for large bacterial fragments in human tissues. Our group is investigating the relationship between arthritis and intestinal bacteria (9). By using a rat model, the arthropathic properties of cell wall fragments of a number of obligate anaerobic bacteria belonging to the major residents of the intestinal flora were described (14, 21–23). Recently, we developed a monoclonal antibody against a bacterial flora-derived peptidoglycan polysaccharide fraction from human feces and found positive staining of rat spleen macrophages (13). Therefore, we have strong support for the presence of bacterial flora-derived products in the spleen. In this study, we analyzed six surgically removed human spleens. Using colorimetric Mur analysis, enzymatic determination of D-lactic acid, and high-pressure liquid chromatography (HPLC) analysis, we demonstrated the presence of substantial amounts of Mur. We concluded that peptidoglycan polymers are present in human spleen.

MATERIALS AND METHODS

Spleen. Six sterile unfixed human spleens were obtained from the pathology department immediately after surgery (SSDZ, Delft, The Netherlands) and kept frozen until use. The spleen was removed from each of five patients (patients 1 through 5) with gastric adenocarcinomas for technical reasons and from patient 6 because of traumatic rupture. Spleen samples were aerobically and anaerobically cultured on blood agar base during 48 h at 37°C. No bacterial growth was observed. The anaerobic plates were cultured in a jar supplied with GasPak generator envelopes with palladium catalyst (Becton Dickinson).

Immunohistologic staining. Immunohistologic staining of the human spleen was performed according by the method of Kool et al. used for the staining of rat spleen (13). The same monoclonal antibody, 2E9 (mouse immunoglobulin G3), recognizing intestinal flora derived peptidoglycan polysaccharides was used.

Spleen extraction. For spleen extraction (Fig. 1), 50 to 100 g of minced spleen in 500 to 1,000 ml of H2O was homogenized in portions by a Virtis homogenizer (Virtis Company) at 10,000 rpm for 30 s. The homogenate was sonicated five times for 1 min each at maximum amplitude (MSE Soniprep 150). Acetic acid (96%) was added to a final concentration of 0.2 M. The extract was incubated at room temperature for 2 h under rotation and was subsequently heated gradually in a water bath to 100°C over a period of 30 min. The extract was centrifuged at 10,000 × g. The volume of the supernatant was reduced by hophilization to 25 to 50 ml and the extract was then centrifuged at 100,000 × g. The supernatants (15-ml portions) were separated by gel filtration on a 275-ml gel bed of Sephadex G-25 (Pharmacia, Uppsala, Sweden). Mur and protein were determined in all fractions by the methods of Hadzija (7) and Bradford (2). The high-molecular-weight fraction was collected and loaded onto a Dowex 50W×4 column (Fluka Chemie AG, Buchs, Switzerland) equilibrated in 0.1 M acetic buffer (pH 4.6) for...
further protein reduction. The volume of the pooled fractions was reduced by lyophilization to 10 ml, and this preparation was dialyzed against 1 dm³ of 1M NaCl and thereafter three times against 3 dm³ of H₂O (bidest). After centrifugation at 100,000 x g, the supernatant was stored and used for determination of Mur (see below). This preparation is further referred to as A. For HPLC analysis of Mur, a further removal of protein was necessary. Therefore, a DEAE-Sepha-
rose CL-6B (Pharmacia) gel bed of 22 ml was washed with 0.5M NaCl followed by a large volume of H₂O. Then 5 ml of preparation A was loaded onto the column and eluted with H₂O until no more protein was detected. A 15-ml Mur-containing fraction was collected after elution with 0.5M NaCl. This 15-ml sample was dialyzed against H₂O, lyophilized, and dissolved in 2 ml of H₂O. This preparation is further referred to as B.

In preparation BMur, protein and total carbohydrates were determined.

## Determination of Mur

### (i) Preparation A

Mur was determined by the method of Hadzija (7), with some modifications (8). In short, samples were hydrolyzed by heating for 2 h at 90°C with an equal volume of 5 M H₂SO₄ and then neutralized with 10 M NaOH. Hydrolyzed and unhydrolyzed samples (100 μl) were incubated with 50 μl of 1 M NaOH at 36°C for 30 min. After the addition of 1 ml of 18.8 M H₂SO₄ (concentrated), samples were heated for 3.5 min at 100°C, rapidly cooled in ice, and then mixed with 10 ml of 0.16 M CuSO₄·5·H₂O in H₂O and 20 ml of 0.09 M p-hydroxydiphenyl in ethanol. The blue color developed to a maximum in 30 min at 30°C. A 570 nm was determined with a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). Solutions containing 0 to 100 μg of Mur (Sigma, St. Louis, Mo.) per ml of H₂O were used as standards. The data are given as the difference in concentration between the hydrolyzed and nonhydrolyzed samples. In this way, only Mur linked to the peptides in peptidoglycan is determined, and disturbing sugars like rhamnose and of course of lactic acid itself, which are determined in the nonhydrolyzed sample, can be excluded. The presence of Mur in preparation A was also determined by measurement of D-lactic acid by the method of Tipper (26). Two milliliters of preparation A was hydrolyzed with 2 ml of 12 M HCl at 90°C for 2 h. The sample was lyophilized and dissolved in 1 ml of H₂O. D-Lactate was determined by enzymatic bioanalysis of D-lactic acid-t-lactic acid (Boehringer Mannheim GmbH, Mannheim, Germany).

### (ii) Preparation B

The presence of Mur was determined with an amino acid amino sugar determination using reverse-phase HPLC techniques as described by Glauer (6). In short, for dansylation of preparation B, 1 ml was hydrolyzed with 500 μl of 12 M HCl for 3 h at 90°C. The sample was frozen, lyophilized, and redissolved in 100 ml of H₂O. This sample was added to 100 μl of 200 mM borate buffer (pH 8.8) and 100 μl of 20 mM dansyl chloride (5-dimethylamino-naphtha-thene-1-sulfonyl chloride; Fluka) in acetone and allowed to react for 2 h at 37°C. The reaction was stopped with 200 mM phosphoric acid. Valine was used as an internal standard, and all other standard amino acids (Sigma) underwent the same treatment.

Separation of dansylated amino acids and amino sugars was accomplished by reverse-phase HPLC. Samples were analyzed with a Pharmacia-LAB 2248 single-pump solvent delivery system and VVM 2141 UV-VIS monitor both connected to a computer working with HPLC Manager software to control the pump, gradient mixer, and UV-VIS detector operating at 330 nm. Integration and analysis of chromatograms was performed with the same software (Pharmacia).

Dansylated samples were separated on a Pharmacia SuperPac Sephasil C₁₈ column (5 μm, 4 by 250 mm). The flow rate used was 1 ml/min, and the buffers used were A (20 mM sodium phosphate [pH 5.25]) and B (60% acetonitrile, 40% 50 mM sodium phosphate [pH 4.0]). At 0, 10, 16, 21.5, 22, and 27 min, the percentages of preparation B were 30, 50, 100, 100, 30, and 30, respectively. Eluted products were detected at 330 nm, the wavelength which is maximally absorbed by the dansyl groups. Internal standard valine and external standards valine and Mur were used for calculating Mur contents of the spleen samples.

## RESULTS

### Immunohistochemistry of the spleen

Red pulp macrophages in the six spleens were immunohistochemically stained. Figure 2 shows an immunohistologic staining of a human spleen with monoclonal antibody 2E9 recognizing intestinal...
flora-derived peptidoglycan polysaccharides. Monoclonal antibody 2-4, recognizing muramyl dipeptide, also stained cells in these spleens (not shown). Negative control antibody NS7 of the same isotype, mouse immunoglobulin G3, and with irrelevant specificity did not stain the spleens. From these positive results, we concluded that bacterial antigens were present in each of the spleens and that these could be used for isolation of a particular bacterial fraction.

**Purification of Mur fraction from spleen.** From all six spleens, preparation A was isolated as described above. The general outlines of the procedure are depicted in Fig. 1. Figure 3 shows the elution pattern after Sephadex G-25 gel filtration of the extracted spleen from patient 1. Protein was found after an elution volume of 95 ml of H₂O. Mur was detected after an elution volume of 85 ml of H₂O and increased to a maximum of 32 μg of Mur per ml. Then it decreased and finally sharply increased to a level much higher than 100 μg/ml. We pooled the fractions between 85 and 130 ml, which represented a relatively high-molecular-weight fraction, and discarded the low-molecular-weight fraction because it contained lactate, which disturbed the colorimetric Mur assay. The pool was loaded on a strong cation-exchange column, Dowex 50W×4, which bound 90% of the protein. After this step, the volume was reduced and the preparation was dialyzed. In a pilot experiment, samples of lysozyme-solubilized cell wall fragments of *Eubacterium aerofaciens* (22), a carbohydrate polymer with...
reduced the protein contents considerably (column with 0.5 M NaCl. DEAE passage of preparation A and B. There are many peaks in the chromatogram, and there was a peak with retention time of 11.3 min in the spleen sample, as depicted in Fig. 4A. To follow up on this peak, we decided to collect the peak with retention time 11.3 min and reanalyze this peak to see if it really has the same retention time as the standard dansyl Mur. Indeed, a major peak of the chromatogram of the collected peak had exactly the same retention time as dansyl Mur (Fig. 4C and D). Finally, we determined Mur in the collected peak by using the colorimetric assay of Hadzija to complete the circle and prove that the spleen samples contain Mur.

Calculations of Mur concentration by using valine as an internal standard gave results in the same range as the other method but may have been hampered by contaminating protein amino acids (Table 2). When the results of Mur concentrations (Tables 1 and 2) are summarized as mean values, then with HPLC 3.3 μmol, with the Hadzija method 1.9 μmol (preparation B) and 2.4 μmol (preparation A), and with the Tipper method 0.8 μmol per spleen (average, 73 g) were found.

**DISCUSSION**

Sen and Karnovsky found 100 pmol of Mur per g of rat liver tissue and extracted muramyl compounds from brain and kidney (20). A daily output of 1 μmol of free diaminopimelic acid, an amino acid specific for muramyl peptides, was found in human urine by Johannsen and Krueger (10). Fox and Fox, however, could not detect Mur in human serum and therefore considered the presence of peptidoglycan- or Mur-containing subunits a matter of dispute (5). In this study, we determined Mur in spleen tissue by three different methods. The spleen was chosen because a previous immunohistochemical study with monoclonal antibodies showed the presence of bacterial antigen in macrophages in the rat spleen (13). The six human spleens used in this study also contained macrophages positively stained by monoclonal antibody 2E9. Specificity of the monoclonal antibody was checked by inhibition of enzyme-linked immunosorbent assays and by inhibition of immunohistologic staining of the spleens. Peptidoglycan polysaccharides were used as antigens.

The isolation procedure was focused on detecting Mur in polymeric peptidoglycan because that is the product which we think is present in the splenic macrophages, given our observations in rats injected with peptidoglycan polysaccharides from *E. aerofaciens* (13). Small muramyl dipeptide administered in vivo in mice, however, is excreted unchanged in the urine within minutes (27). The practical advantage of this assumption is that during the extraction and purification, the low-molecular-weight fraction could be discarded. In this respect, our study differed from the studies of Sen and Karnovsky (20), who were looking for low-molecular-weight muropeptides. Before and after homogenization of the spleen tissues, sterility was checked by cultivating the homogenate on a blood agar plate. No contamination could be detected. Acetic acid extraction was used to hydrolyze the lysosomes in the spleen homogenates in which the peptidoglycan might be located (19). After this, the aim was to remove as much protein as possible while keeping the carbohydrate fraction. In the end, we succeeded in isolating such a fraction containing a considerable amount of Mur. Mur was used as a marker for the presence of polymers of peptidoglycan because Mur is considered to be unique for peptidoglycan.

We used three determination methods for Mur. (i) The method of Hadzija (7) (modified by Hazenberg et al. [8]) is based on the colorimetric determination of the lactyl group after acid hydrolyzes to remove peptides in peptidoglycan and treatment with NaOH to hydrolyze the ether bond of the lactyl group in Mur.

(ii) The method of Tipper (26) was also used. D-Lactic acid
is specific for bacteria (24), and the covalent ether bond of \(\alpha\)-lactic acid in Mur is very specific for this molecule and therefore for peptidoglycan. We adopted the method of Tipp, in which lactate dehydrogenase is used for the detection of \(\alpha\)-lactic acid in peptidoglycan liberated after acid and alkali hydrolysis. Indeed, \(\alpha\)-lactate was found in our \textit{E. aerofaciens} cell wall fragment preparation after consecutive acid and alkali hydrolysis (data not shown).

(iii) Because both methods for determination of Mur are based on detection of the (\(\alpha\)) lactyl group, a third method not dependent on the presence of lactic acid was used. The HPLC analysis of amino sugars was carried out by a modified procedure developed by Glauner (6), who studied the composition of peptidoglycan of \textit{Escherichia coli} extensively. After collection of the peaks with a retention time of 11.3 min (Fig. 4), we were able to determine Mur in this fraction with the Hadzija method. We conclude that in the human spleen Mur and therefore peptidoglycan is present not as small muramyl peptides as were previously looked for but as larger molecules probably stored in spleen macrophages.

Peptidoglycan has many biological effects in vivo; they include sleep induction (16), complement activation (30), adjuvant activity (4), and many other immunostimulatory effects such as antibody production (28) and T-cell-mediated arthritis induction (9, 14, 21–23). In vitro peptidoglycans are able to stimulate human monocytes to produce tumor necrosis factor alpha and interleukin-1 and inhibition or activation of monocyte migration (18, 25). From the results presented in this report and results from other investigators, it can be concluded that our body tissues are continuously exposed to these bacterial products which in some cases might lead to biological activities in vivo. If so, this in its turn might provide a clue to inflammation with unknown etiology, such as rheumatoid arthritis and reactive arthritis. Presently we are investigating the biological properties of this peptidoglycan.

ACKNOWLEDGMENTS

We gratefully thank Tar van Os and Marjoe van Everdingen-Quartel for excellent assistance in preparation of the manuscript.

REFERENCES


