BIOLOGICAL PROPERTIES OF CELL WALL MUCOPEPTIDE OF HEMOLYTIC STREPTOCOCCI*

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(Received for publication 27 February 1969)

Although the mucopeptide[1] of hemolytic streptococci is not chemically similar to the endotoxins of Gram-negative bacteria, both substances have several biological properties in common. For example, streptococcal mucopeptide, when injected into mice, either intraperitoneally or intravenously, enhances the resistance to subsequent challenge with virulent Group A streptococci. Mucopeptide produces fever in the rabbit and may initiate shock and death (1). A necrotic skin lesion develops in rabbits after local injection of mucopeptide (2). In this report, the febrile response after injection of mucopeptide has been studied in greater detail, and mucopeptide has been used to prepare and provoke the local Shwartzman reaction. Special attention has been given to pathologic alterations which occur in the myocardium of rabbits after the intravenous administration of mucopeptide, a finding which was reported earlier as a preliminary observation (3).

Materials and Methods

Streptococcal Strains.—Group A, Type 6 strain S 43/100/14, Group B strain 090 R, and Group L strain SHC 16 were used for the preparation of the mucopeptide. The Groups A and B strains were obtained from Dr. Rebecca Lancefield, The Rockefeller University, New York; the Group L strain was from the Streptococcus Reference Laboratory, Institute of Epidemiology and Microbiology, Prague.

Mucopeptide and Carbohydrate.—Mucopeptide and carbohydrate were prepared by extraction of cell walls with formamide as described by Fuller (4) and modified by Krause and McCarty (5).

Throughout the whole procedure for the preparation of mucopeptide and carbohydrate, from the growth of the bacteria to the final lyophilization of the products, care was taken to

* The preliminary studies of this work were begun at the time the senior author was a visiting investigator in the laboratory of Dr. Richard M. Krause, Washington University School of Medicine, St. Louis, Missouri, and were supported at that time by United States Public Health Service, National Institutes of Health Grant HE 08027, and were conducted in part under the sponsorship of the Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiology Board, and was supported by the Office of the Surgeon General, Department of the Army, Washington, D.C.

[1] Other terms used in the literature for mucopeptide include peptidoglycan and murein.

avoid contamination by extraneous pyrogens. All glassware and containers were heated to 180°C for 2 hr; only pyrogen-free water was used to prepare reagents.

Cell walls were prepared from streptococci grown for 18 hr in Todd-Hewitt broth. The culture was centrifuged at 4°C and the bacterial sediment was washed three times with distilled water (pyrogen-free water used throughout). The bacteria were then resuspended in distilled water in a volume equal to 1/100 of the broth culture volume, and were disrupted in a Mickle disintegrator using Ballotini No. 12 glass beads. The disintegrated material was centrifuged at 6000 rpm (MSE centrifuge, rotor no. 9180), and the sediment was washed three times with distilled water.

TABLE I

Chemical Composition of Streptococcal Mucopeptide Prepared from Group A Strain S43/100/14

Component	μmole/mg	μg/mg
N-Ac-Muramic Acid	0.602	176.55
N-Ac-Glucosamine	0.772	170.77
Lysine	0.822	120.17
Glutamic Acid	0.779	114.63
Glycine	0.081	6.08
Alanine	3.145	280.22
Histidine	0.010	1.55
Arginine	0.005	0.87
Aspartic Acid	0.014	1.86
Threonine	0.010	1.19
Serine	0.014	1.47
Tyrosine	0.024	4.35
Phenylalanine	0.029	4.79
Ammonia	1.127	19.16
Total Recovery		903.66

All values are given in terms of the anhydrous ash-free mucopeptide. The lyophilized streptococcal mucopeptide was 6.25% moisture and 2.30% ash. The μg/mg values for the amino sugars have been calculated to include the acetylation of these sugars in the native state.

The ammonia values were corrected for the amounts of ammonia contributed by decomposition of the amino sugars. The amount contributed by degradation of trace amino acids such as serine has not been included.

The authors are grateful for the assistance of Mr. Henry Lackland, The Rockefeller University, who performed these determinations.

Cell walls were treated with ribonuclease and deoxyribonuclease as described by Freimer for preparation of protoplast membranes (6), followed by trypsin treatment (5). After dialysis against distilled water they were lyophilized. The cell walls were extracted twice with hot formamide at 180°C to remove the group-specific carbohydrate from the mucopeptide residue. The carbohydrate was recovered from the extract by the described procedure and lyophilized (5). The particulate mucopeptide residue was washed extensively with distilled water, dialyzed, and lyophilized.

Chemical Analysis of Mucopeptide.—Glucosamines and amino acids were determined with the Beckman amino acid analyzer by the methods of Spackman, Stein, and Moore (7), as described by Karakawa et al (8). Rhamnose was determined by the method of Dische and Shettles (9).

In any study directed at the biological properties of a substance, such as mucopeptide, which is obtained in part by cell fractionation procedures, it is important to have accurate information on the total chemical composition. Special care was taken to avoid the accumulation of extraneous material throughout the preparation of the several lots of mucopeptide employed here. A typical chemical analysis of a representative batch of mucopeptide is given in Table I. Amino acids and amino sugars account for 88.5% of the material. Rhamnose content was less than 2%.

Solubilization of Mucopeptide.—One mg of particulate mucopeptide per ml of saline was solubilized by ultrasonic treatment in an MSE disintegrator, 20 kc, for 30 min. Mucopeptide solubilized in this fashion was used for the biologic studies and as the antigen in precipitin tests for detection of mucopeptide antibodies.

Antiserum to Mucopeptide.—Antisera were prepared by immunizing rabbits with particulate mucopeptide isolated from cell walls of Group A, Type 6 streptococcus. The following immunization scheme was used: 1st wk, 2.5 mg mucopeptide in incomplete Freund's adjuvant into each foot pad. Each week from the 5th–10th wk, an intraperitoneal injection of 10 mg particulate mucopeptide was followed the next day by an intravenous dose of 10 mg mucopeptide. 10 days after the last injection, ring and capillary tests were used to detect antibodies. One of the four immunized rabbits produced sufficient antibodies to mucopeptide, so the serum could be used in experimental procedures.

Endotoxin.—A Difco product prepared from *Escherichia coli* 0127:B8 was used.

E. coli Lipopolysaccharide.—Lipopolysaccharide, prepared from *E. coli* 12408 strain, was kindly supplied by Dr. E. Work, Twyford Laboratories, Ltd., London.

Cortisone.—The cortisone-acetate is a product of Continental Pharma, Brussels.

Rabbits.—White rabbits weighing 1.7–1.9 kg, kept at a constant temperature and fed on standard diet without antibiotic additive, were used for all aspects of these studies.

Determination of Febrile Response in Rabbits.—Rabbits were employed which had a rectal temperature variation less than 0.3°C during 6 hr of observation on the day preceding the experiment. The material to be tested was injected into the marginal ear vein, and the temperature was measured from the rectum.

Unless stated otherwise in the protocols which follow, all preparations examined for pyrogenic properties were tested in at least 3 rabbits. Average values were used to plot the fever-response curves.

RESULTS

Pyrogenic Properties of Mucopeptide.—In previous experiments, particulate mucopeptide, recovered as an insoluble residue after extraction of cell walls with hot formamide, was solubilized by ultrasonic treatment and administered intravenously to produce fever in rabbits (1). The dose fever-response curves after injection of solubilized mucopeptide are now presented in detail.

Mucopeptide, after treatment for 30 min in an MSC ultrasonic disintegrator, is referred to as soluble mucopeptide, although this material is heterogeneous with respect to size. For example, 49% of the disrupted mucopeptide is sedimented at 25,000 RCF (relative centrifugal force) for 1 hr, and 62% is sedimented at 100,000 RCF.

The temperature elevations after injection of soluble mucopeptide are depicted in Fig. 1a. A reproducible febrile response which persisted for 12–24 hr was obtained with 3.2 μg. When less than this dose was employed, the fever was not only lower, but often delayed for 3–5 hr after the injection. Mucopeptide,

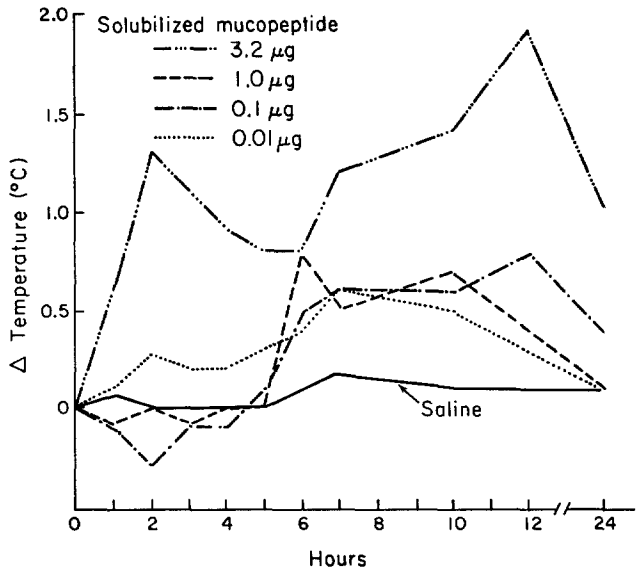


FIG. 1*a*. Febrile responses after intravenous injections of different doses of solubilized mucopeptide.

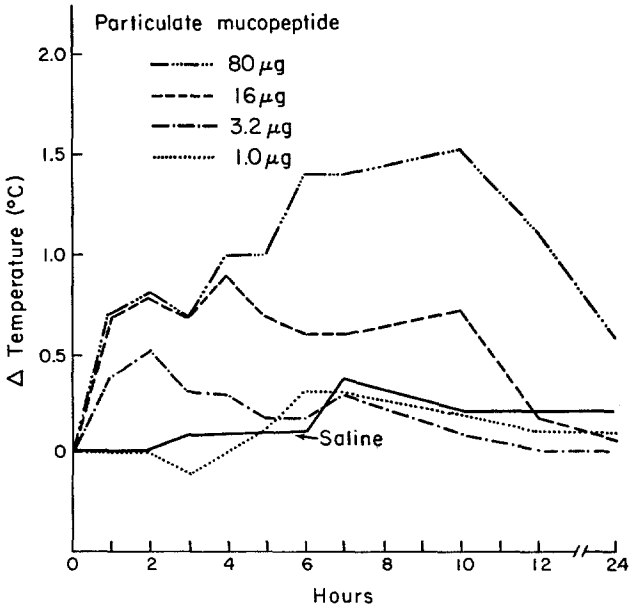


FIG. 1*b*. Febrile responses after intravenous injections of different doses of particulate mucopeptide. Each dose was injected into 3 rabbits. Each curve was obtained by averaging the temperature values of three rabbits.

which has not been subjected to ultrasonic treatment is particulate in nature and is readily sedimented at 5000 RCF for 30 min. It is much less effective in producing fever. Dose-fever response curves after injection of particulate mucopeptide are depicted in Fig. 1b. 80 μg of particulate mucopeptide was required to produce a fever response comparable to that achieved with 3.2 μg of sonically disrupted mucopeptide.

Studies to determine optimal particle size of the sonically disrupted mucopeptide for the production of fever were inconclusive. It was clear, however, that

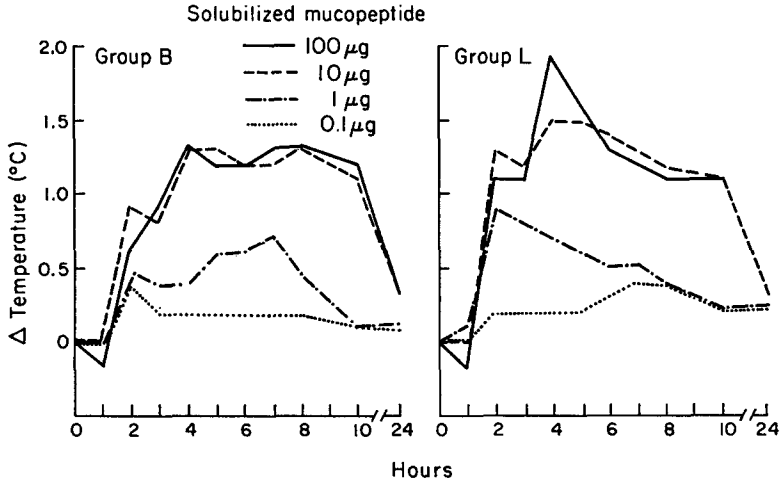


FIG. 2. Febrile responses after intravenous injections of different doses of Groups B and L solubilized mucopeptide. Each dose was injected into 3 rabbits. Each febrile response curve is the average for 3 rabbits.

fever was produced by mucopeptide particles which were not sedimented at either 25,000 RCF or 100,000 RCF for 1 hr.

A large body of information suggests that the mucopeptides of several different serologic groups of hemolytic streptococci are similar chemical compounds (5, 8). It was anticipated, therefore, that the mucopeptides of these other groups would also have pyrogenic activity. The dose-fever response curves for Groups B and L solubilized mucopeptides are presented in Fig. 2. 10 μg elicited a reproducible febrile response, although injection of as little as 1 μg was sufficient to produce detectable fever.

The febrile response to mucopeptide, like that to endotoxin, can be readily diminished or eliminated by pretreatment with cortisone. Four rabbits were given 10 mgm of cortisone intramuscularly twice a day for 2 days. After the last dose, 2 mg of soluble mucopeptide was injected intravenously. During the subsequent 10 hr, no fever was noted while control rabbits responded in the usual fashion.

One interpretation of the febrile response after injection of streptococcal mucopeptide is that it may have become contaminated with an extraneous pyrogen during preparation. Against this possibility is the fact that the group-specific carbohydrate, isolated from the same cell wall extract which served as the source of mucopeptide, is neither pyrogenic nor toxic. For example, the temperature of rabbits remained within 0.2°C of control levels after intravenous injection of 2.0 mgm of Group A carbohydrate. The possibility that the mucopeptide is toxic because of an extraneous substance also appears unlikely as a

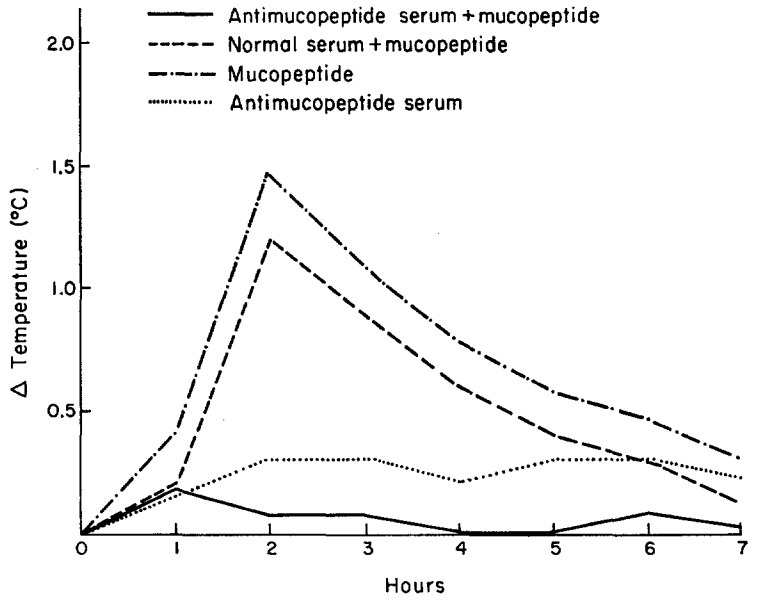


FIG. 3. Neutralization of the pyrogenic activity of solubilized mucopeptide by antiserum to mucopeptide. See text for description of materials injected. Each febrile response curve is the average for 3 rabbits.

result of additional experiments which have made use of mucopeptide antiserum. The addition of antiserum to solubilized mucopeptide eliminated pyrogenic activity.

Optimal proportions of mucopeptide and antiserum were determined by prior precipitin reactions. To each 0.9 ml of antiserum to mucopeptide was added 0.1 ml of saline containing 50 μg of solubilized mucopeptide and the mixture incubated for 1 hr at 20°C and then for 18 hr at 4°C. The immune precipitate was centrifuged at 2000 rpm for 30 min and the pyrogenic activity of the supernatant fluid was tested in rabbits. Each rabbit received 1.0 ml of this supernatant. Control rabbits received an equal volume of supernatant

fluids of the following mixtures which were prepared according to the following proportions: 0.9 ml of normal rabbit serum and 0.1 ml of solubilized mucopeptide; 0.9 ml of saline and 0.1 ml of mucopeptide; and 0.9 ml of antiserum and 0.1 ml of saline. The pyrogenic responses of the rabbits injected with each of the

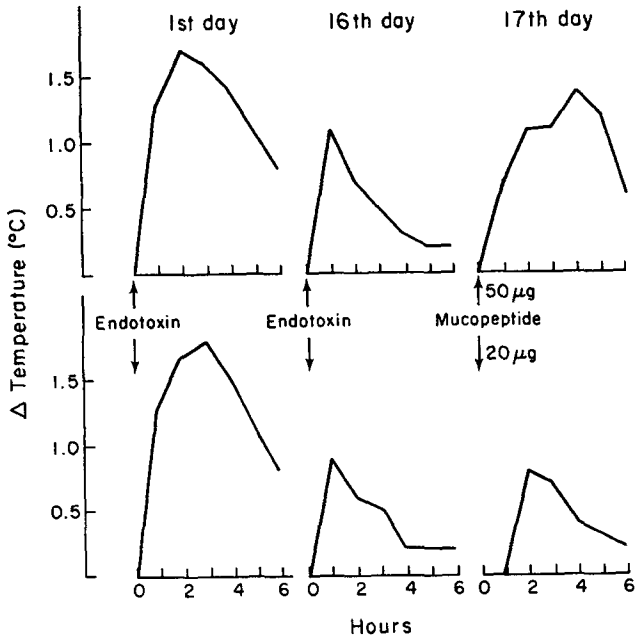


FIG. 4. Induction of tolerance with 16 daily intravenous injections of 0.5 μg of *E. coli* lipopolysaccharide.

Upper frame. The febrile response curves represent the average temperatures for 3 rabbits on day 1 and day 16 after the administration of the daily dose of endotoxin. 50 μg of solubilized mucopeptide was injected intravenously on the 17th day.

Lower frame. Similar febrile response curves for the average of 3 other rabbits after injections of endotoxin. In this case 20 μg of solubilized mucopeptide was injected intravenously on the 17th day.

supernatant fluids are recorded in Fig. 3. The supernatant fluid from the mucopeptide-antimucopeptide mixture did not produce fever, whereas the supernatant fluids of mucopeptide alone, and normal rabbit serum and mucopeptide produced a predictable and typical febrile response for this dose of mucopeptide. Similar results were obtained when the same antiserum prepared with Group A mucopeptide was used in conjunction with Group B mucopeptide. These results indicate that the pyrogenic element in solubilized mucopeptide is precipitated by mucopeptide antiserum, and argue against the possibility that the pyrogenic

property of mucopeptide is the result of contamination with ubiquitous pyrogens.

An extensively studied aspect of the fever induced in rabbits by endotoxin is the tolerance which develops with repeated injections (10). Because endotoxins of Gram-negative bacteria and streptococcal mucopeptide are chemically quite different substances, it was anticipated that rabbits which were made tolerant to endotoxin of Gram-negative bacteria would nevertheless respond to subsequent injection of streptococcal mucopeptide. Rabbits were made tolerant to the lipopolysaccharide of *E. coli* by daily injections of 0.5 μg for 16 days. The

TABLE II

Local Shwartzman Reactions Obtained with Solubilized Mucopeptide and E. coli Endotoxin

Intradermal injection	Intravenous injection	No. of rabbits	No. of rabbits with reaction
mg	*mg*		
Streptococcal mucopeptide	Streptococcal mucopeptide		
0.4	0.4	3	1
0.4	2.0	2	1
2.0	0.4	3	1
2.0	2.0	3	2
Streptococcal mucopeptide	*E. coli* endotoxin		
0.4	0.1	3	1
2.0	0.1	3	1
E. coli endotoxin	Streptococcal mucopeptide		
0.1	0.4	3	2
0.1	2.0	5	5

The interval between the intradermal and intravenous injections was 24 hr. The mucopeptide had been solubilized by ultrasonic treatment. Difco *E. coli* endotoxin was used.

following day they were challenged with solubilized mucopeptide. A control group of rabbits were injected daily for 16 days with saline and on the following day challenged with mucopeptide. The results are presented in Fig. 4. Although 20 μg of mucopeptide did not break the tolerance of the endotoxin-tolerant rabbits, 50 μg of mucopeptide was sufficient to do so. Either the 20 μg or the 50 μg dose of mucopeptide elicited a prompt, brisk febrile response in control rabbits. An attempt was made to do the reciprocal experiments in which endotoxin was given to break tolerance produced with mucopeptide. The results were inconclusive, however, because tolerance after repeated injections of mucopeptide was inconstant.

The Local Shwartzman Phenomenon.—One of the most thoroughly studied biological properties of endotoxin is the capacity to prepare and provoke the general and local Shwartzman phenomena (11). Because there is a resemblance

between the biological properties of mucopeptide thus far described and those of endotoxin of Gram-negative bacteria, attempts were made also to produce the Shwartzman phenomenon with mucopeptide. Two intravenous injections of mucopeptide, spaced 24 hr apart, failed to produce the generalized Shwartzman phenomenon. Various dosage schedules from 0.4–10 mg were employed for both the preparatory and the provocative injections. Autopsy search of rabbits revealed no evidence of bilateral cortical necrosis of the kidney. For reasons which remain unexplained, 2 of the 12 rabbits tested died a few min after the provocative dose of mucopeptide.

Success was achieved when mucopeptide was used to prepare and provoke the localized Shwartzman phenomenon. The injection schedule, the number of rabbits employed, and the number of rabbits which exhibited a localized reaction are presented in Table II. Endotoxin could be substituted for mucopeptide as the preparative injection, as well as the provocative injection. A picture of the localized Shwartzman reaction is presented in Fig. 5. In additional studies, intravenous injection of either Group B or Group L solubilized mucopeptide was used to provoke the localized Shwartzman reaction in rabbits prepared with an intradermal injection of *E. coli* endotoxin.

Intravenous injections of particulate mucopeptide did not provoke a local reaction in rabbits which had received intravenous injections of endotoxin. Group A carbohydrate, isolated from the same formamide extract as the mucopeptide, was ineffective in eliciting the Shwartzman phenomenon.

Other Toxic Effects of Mucopeptide.—Because of the death of some of the rabbits after intravenous administration of mucopeptide, histologic studies were undertaken to determine possible pathologic alterations.

Rabbits were injected intravenously with 2 mg, 80 μg, and 1 μg of solubilized mucopeptide. Each dose of mucopeptide was injected into 5 rabbits. Five control rabbits were injected with saline which had been exposed in a control fashion to the sonic oscillator. Rabbits were killed by decapitation 48 hr after injection. Gross inspection revealed no significant alteration of the organs.

Although histologic examination of the liver, spleen, and skeletal muscle revealed minimal nonspecific changes, extensive alterations were observed in the heart. While the extent of these changes depended upon the size of the dose of mucopeptide, the character of the pathology in each instance was the same.

Three of five rabbits injected intravenously with 2 mg of solubilized mucopeptide had the extensive pathologic alterations in the right ventricle which are depicted in Fig. 6, *a*, *b*, *c*. There is intense myocardial degeneration obviously involving the cardiac muscle cells. Various stages of degeneration and necrosis are observed. Extrusion of nuclei has occurred. Occasional nuclei of the cardiac cells are in metaphase, suggesting initiation of regeneration. These alterations are not seen in the smooth muscle of the coronary vessels. There is a striking infiltration of polymorphonuclear leukocytes into the tissue. Although these

alterations are most extensive in the right ventricle, changes are observed elsewhere in the myocardium also. Similar changes, but somewhat less marked, were seen in the hearts of the other two rabbits injected with 2 mg of solubilized mucopeptide.

Three of the five rabbits injected with 80 μg of mucopeptide and three of the five rabbits injected with 1 μg of mucopeptide had a carditis similar to that described above, but the extent was proportionately less. For example, in the case of rabbits which were injected with 1 μg of solubilized mucopeptide, these alterations were observed approximately once in every 10 microscopic fields at ×350 magnification. There were no changes in the hearts of the five rabbits injected with saline.

Microscopic examination of five rabbits injected with particulate mucopeptide revealed minimal pathologic alterations in the heart. It is conceivable that the particulate material is cleared by passage through the pulmonary-capillary bed and does not reach the myocardium. Additional rabbits were tested to gain some information on the particle size of the material which produced the lesions. One mg/ml was solubilized by ultrasonic treatment and aliquots centrifuged at 25,000 RCF and 100,000 RCF for 1 hr. Rabbits were injected with 1 ml of the supernatants of each aliquot. The extent and severity of the lesions were similar to those observed in rabbits injected with solubilized mucopeptide which had not been centrifuged.

In another series of experiments rabbits were examined up to 10 days after injection of mucopeptide. Six rabbits were injected with 100 μg, and six with 10 μg of solubilized mucopeptide. Three rabbits from each dosage group were autopsied at 3 days and 10 days. The microscopic findings in the hearts at 10 days were similar to those observed at 3 days. Control rabbits revealed negative findings. Lesions were not observed in skeletal muscle.

The basis for these pathologic alterations in the heart after intravenous injection of mucopeptide remains unexplained. In studies currently underway, mucopeptide did not possess direct toxicity for the isolated perfused rabbit heart.[2]

DISCUSSION

The biological properties of streptococcal mucopeptide described in detail here augment the current information on its chemical nature. The general outline for the structure of bacterial mucopeptides has been now largely clarified (12, 13, 14). These substances consist of repeating units of *N*-acetyl glucosamine and *N*-acetyl-muramic acid. In the case of streptococcal mucopeptide, tetrapeptides composed of L-alanine, D-glutamic acid, glycine, and D-alanine are linked to the hexosamine polymer through the carboxyl group of the muramic acid, and in turn, the tetrapeptides of adjacent hexosamine polymers are covalently

[2] Rašková, H., K. Mašek, and J. Rotta. Personal communication.

cross-linked by L-alanyl-L-alanine to form a matrix (15). The mucopeptides of the various bacteria differ primarily in the amino acid content of the tetrapeptides and the cross-link peptides. The mucopeptides of certain Gram-negative organisms, for example, contain diaminopimelic acid instead of lysine in the tetrapeptide, and the cross-links of staphylococci are peptides of glycine (12). Thus, it is clear that the mucopeptides are chemically unlike the endotoxins of Gram-negative bacteria. Although it is known that the endotoxins consist of the lipopolysaccharide moiety of the somatic O antigen, there is much which is as yet uncertain about the chemical structure (16).

A number of the special biological and toxic properties of the Gram-negative endotoxins have now been described for streptococcal mucopeptide. The febrile response in rabbits after intravenous injection of mucopeptide has been presented in detail here. Intraperitoneal or intravenous administration of streptococcal mucopeptide in mice enhances resistance to subsequent challenge with virulent Group A streptococci (1). Abdulla and Schwab have shown dermal necrosis after intradermal injection of streptococcal mucopeptide into the skin of rabbits (2). Finally, in the studies reported here, streptococcal mucopeptide was successfully employed to prepare and to provoke the local Shwartzman phenomenon.

These properties of streptococcal mucopeptide differ to some extent from those of a streptococcal endotoxin described by Stetson (17). This latter material was obtained from the supernatant fluid of disrupted Group A streptococci which had been centrifuged to remove the cell walls. While the chemical nature of Stetson's streptococcal endotoxin is not known, it is unlikely on the basis of the method of preparation that it was derived from cell walls, or that it is identical with the mucopeptide described here. Furthermore, the two products appear to have some differences in their biologic effects. The streptococcal endotoxic material prepared by Stetson induced a state of tolerance to the pyrogenic effect which could be broken with subsequent Gram-negative endotoxin, while the induced tolerance achieved with streptococcal mucopeptide was inconstant. The local Shwartzman reaction appears in about 3 hr after the intravenous injection of the streptococcal endotoxin which is also the case for the Gram-negative endotoxins. In contrast, skin hemorrhage and necrosis appear in approximately 30 min after intravenous injection of mucopeptide. Such an accelerated reaction has also been reported after intravenous injection of nonspecific materials, such as starch and agar (11), into rabbits with suitable prepared skin sites.

It has yet to be learned if the toxic properties of mucopeptide are due to intrinsic toxicity of the substance or due to hypersensitivity or immunity. Such a dilemma persists in regard to Gram-negative bacterial endotoxin. Stetson has argued that the endotoxins possess biologic activity only by virtue of their antigenicity and that the toxic properties are not manifestations of an intrinsic toxicity but a reflection of an immune or a hypersensitivity response. Such an argument, supported by the findings of a number of investigators, has been summarized recently (18). Needless to say, this view is not universally held. Kim and Watson, for instance, have demonstrated

that piglets, obtained by hysterectomy and deprived of colostrum, possessed no immunoglobulins, but were nevertheless sensitive to the lethal effects of endotoxin (19). Such data argue in favor of an intrinsic toxic property of endotoxin which is not dependent upon acquired immunity.

These comments on the role of immunity in the toxicity of endotoxin may be relevant to a consideration of the toxicity of mucopeptide. Cromartie, Schwab, and Ohanian have suggested that the toxic properties of cell walls are due to the persistence of fragments as durable material in the tissue, thus leading to focal lesions. It is argued that the mucopeptide is the toxic moiety of the cell wall fragments (20, 21). On the other hand, there is now accumulating evidence that the mucopeptides are antigenic and that immune sera produced by injection of bacterial vaccines contain mucopeptide antibodies (8, 22–26). Immunochemical data suggest that such antisera contain antibodies to the peptide moiety and antibodies to the hexosamine polymer of the mucopeptide (22). Furthermore, immunologic cross-reactivity among the mucopeptides of several different Gram-positive bacteria, including staphylococci and hemolytic streptococci, has been observed. The immunochemical basis for this cross-reactivity is due to the fact that the mucopeptides of these two organisms contain chemically similar tetrapeptides (8). It is reasonable to presume that additional studies will reveal widespread immunologic cross-reactivity among the bacterial mucopeptides. Thus, prolonged and continuous exposure of animals and man to bacteria provides a means for the natural acquisition of sensitivity to these substances. It remains to be determined if any of the toxic reactions are either diminished or eliminated when mucopeptide is injected into germfree animals. Such an outcome would suggest that the pathogenesis of the toxic manifestations depends in part upon acquired immunity.

In the studies reported here, an extensive carditis, more pronounced in the right ventricle, was the most dramatic toxic effect after intravenous administration of streptococcal mucopeptide into rabbits. 2 and 3 days after injection, necrosis of myocardial cells and infiltration with leukocytes was observed. Examination of rabbits 10 days after injection revealed a persistence of this carditis. These findings in the heart are similar in some respects to those seen in the myocardium of mice after intravenous or intraperitoneal injection of sonically disrupted Group A streptococci or sonically disrupted Group A streptococcal cell walls (21, 27). It is suggested by these authors that in this case, also, the mucopeptide is the toxic moiety of the cell wall fragments, but the proof is indirect, because cell walls, and not mucopeptide alone were injected. Nevertheless, the evidence obtained by immunofluorescent techniques is convincing that cell wall fragments persist for prolonged periods in the heart after injection of the material into the peritoneum (21).

There does appear to be a major difference between the dose of mucopeptide required to produce cardiac lesions as described here for rabbits and the dose of cell wall fragments required to produce cardiac lesions in mice (27). 80 μg of sonically disrupted mucopeptide was sufficient to produce carditis in rabbits,

whereas the sonically disrupted Group A streptococci which was injected intravenously into mice contained at least 200–400 μg of cell walls (27). These differences in dosage which are required to produce the cardiac lesions may be due to the quite different nature of the materials employed, or reflect variability between the two species. The relationship of this mucopeptide-induced carditis to the other toxic manifestations of mucopeptide remains to be determined.

Note added to proof: The authors have been informed by Dr. W. W. Karakawa and Dr. P. Maurer that tanned red cells undergo lysis upon exposure to streptococcal mucopeptide. Dr. M. Rýe, Dr. H. Rašková, and Dr. J. Rotta have recently found that thrombocytes are lysed by mucopeptide. It is conceivable that the toxic manifestations of mucopeptide described here may be related to this lytic property.

SUMMARY

Several of the toxic properties of streptococcal mucopeptide have been studied in detail. Intravenous injection of as little as 1 μg of mucopeptide, solubilized by ultrasonic treatment, elicits a reproducible febrile response. Rabbits which are made tolerant to *Escherichia coli* endotoxin are only partially tolerant to the subsequent injection of streptococcal mucopeptide. Soluble mucopeptide was successfully employed to prepare and provoke the localized Shwartzman reaction.

Intravenous injection of 80 μg of solubilized mucopeptide leads to diffuse cellular infiltration as well as focal areas of myocardial necrosis, surrounded by inflammatory cells.

The authors are pleased to acknowledge the helpful suggestions of Dr. Richard M. Krause throughout the course of this work, and his guidance during the preparation of the manuscript.

The authors are grateful for the capable technical assistance of Mrs. R. Bicová, Mrs. D. Stržinková, Miss J. Žirovnická, and Mrs. Z. Rudová.

BIBLIOGRAPHY

1. Rotta, J., T. J. Prendergast, W. W. Karakawa, C. K. Harmon, and R. M. Krause. 1965. Enhanced resistance to streptococcal infection induced in mice by cell wall mucopeptide. *J. Exp. Med.* **122**:877.
2. Abdulla, E. M., and J. H. Schwab. 1966. Biological properties of streptococcal cell wall particles. III. Dermonecrotic reaction to cell wall mucopeptide. *J. Bacteriol.* **91**:374.
3. Raška, K., and J. Rotta. 1967. *In* Streptokokové Nákazy A Jejich Následky. Státní Zdravotnické Nakladatelství, Prague. 197.
4. Fuller, A. T. 1938. The formamide method for the extraction of polysaccharide from hemolytic streptococci. *Brit. J. Exp. Pathol.* **19**:130.
5. Krause, R. M., and M. McCarty. 1961. Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of Groups A and A-variant streptococci. *J. Exp. Med.* **114**:127.
6 Freimer, E. H. 1963. Studies of L forms and protoplasts of Group A streptococci.

II. Chemical and immunological properties of the cell membrane. *J. Exp. Med.* **117**:377.

7. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* **30**:1190.
8. Karakawa, W. W., D. G. Braun, H. Lackland, and R. M. Krause. 1968. Immunochemical studies on the cross-reactivity between streptococcal and staphylococcal mucopeptide. *J. Exp. Med.* **128**:325.
9. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses and a spectrophotometric micromethod for their determination. *J. Biol. Chem.* **135**:595.
10. Beeson, P. B. 1947. Tolerance to bacterial pyrogens. II. Role of the reticuloendothelial system. *J. Exp. Med.* **86**:39.
11. Shwartzman, G. 1937. Phenomenon of Local Tissue Reactivity. Paul B. Hoeber, Inc., New York.
12. Salton, M. R. J. 1964. The Bacterial Cell Wall. Elsevier Publishing Co., Amsterdam. 133.
13. Perkins, H. R. 1963. Chemical structure and biosynthesis of bacterial cell walls. *Bacteriol. Rev.* **27**:18.
14. Park, J. T. 1967. The mechanism by which penicillin causes conversion of bacterial cells to spheroplasts. *In* Microbial Protoplasts, Spheroplasts, and L-forms. L. B. Guze, editor. The Williams and Wilkins Co., Baltimore. 52.
15. Petit, J. F., E. Munoz, and J. M. Ghuysen. 1966. Peptide crosslinks in bacterial cell wall peptidoglycan studied with specific endopeptides from *Streptomyces albus*. *J. Biochem.* **5**:2764.
16. Ludoritz, B., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related *Enterobacteriaceae*. *Bacteriol. Rev.* **30**: 192.
17. Stetson, C. A. 1965. The endotoxic properties of lysates of Group A hemolytic streptococci. *J. Exp. Med.* **104**:921.
18. Stetson, C. A. 1965. Role of hypersensitivity in reaction to endotoxin. *In* Bacterial and Mycotic Diseases of Man. R. Dubos and J. Hirsch editors. J. B. Lippincott Co., Philadelphia. 658.
19. Kim, Y. B., and D. W. Watson. 1966. Role of antibodies in reactions to gram-negative bacterial infections. *Ann. N.Y. Acad. Sci.* **133**:727.
20. Ohanian, S. H., and J. H. Schwab. 1967. Persistence of Group A streptococcal cell walls related to chronic inflammation of rabbit dermal connective tissue. *J. Exp. Med.* **125**:1137.
21. Ohanian, S. H., J. H. Schwab, and W. J. Cromartie. 1969. Relation of rheumatic-like cardiac lesions of the mouse to localization of Group A streptococcal cell walls. *J. Exp. Med.* **129**:37.
22. Karakawa, W. W., and R. M. Krause. 1966. Studies on the immunochemistry of streptococcal mucopeptide. *J. Exp. Med.* **124**:155.
23. Karakawa, W. W., H. Lackland, and R. M. Krause. 1966. An immunochemical analysis of bacterial mucopeptides. *J. Immunol.* **97**:797.
24. Hisatsune, K., S. J. DeCourey, and S. Mudd. 1967. Studies on the carbohydrate-

peptide fraction of centrifugal supernatant of *Staphylococcus aureus* cultures. *Biochemistry.* **6:**586.

25. Abdulla, E. M., and J. H. Schwab. 1965. Immunological properties of bacterial cell mucopeptides. *Proc. Soc. Exp. Biol. Med.* **118:**399.
26. Gotschlich, E. C., and T. Y. Liu. 1967. Structural and immunological studies on the pneumococcal C polysaccharide. *J. Biol. Chem.* **242:**463.

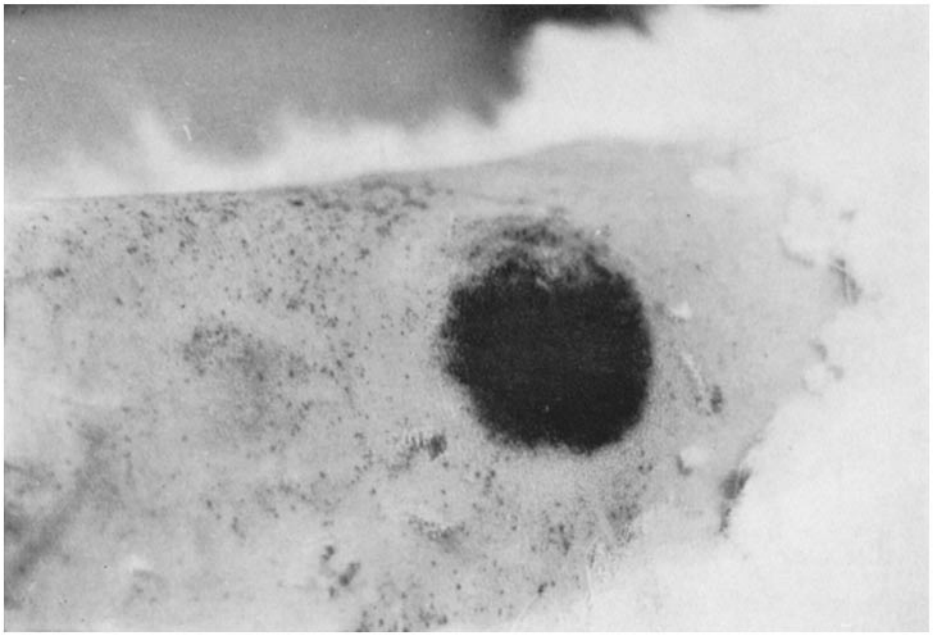
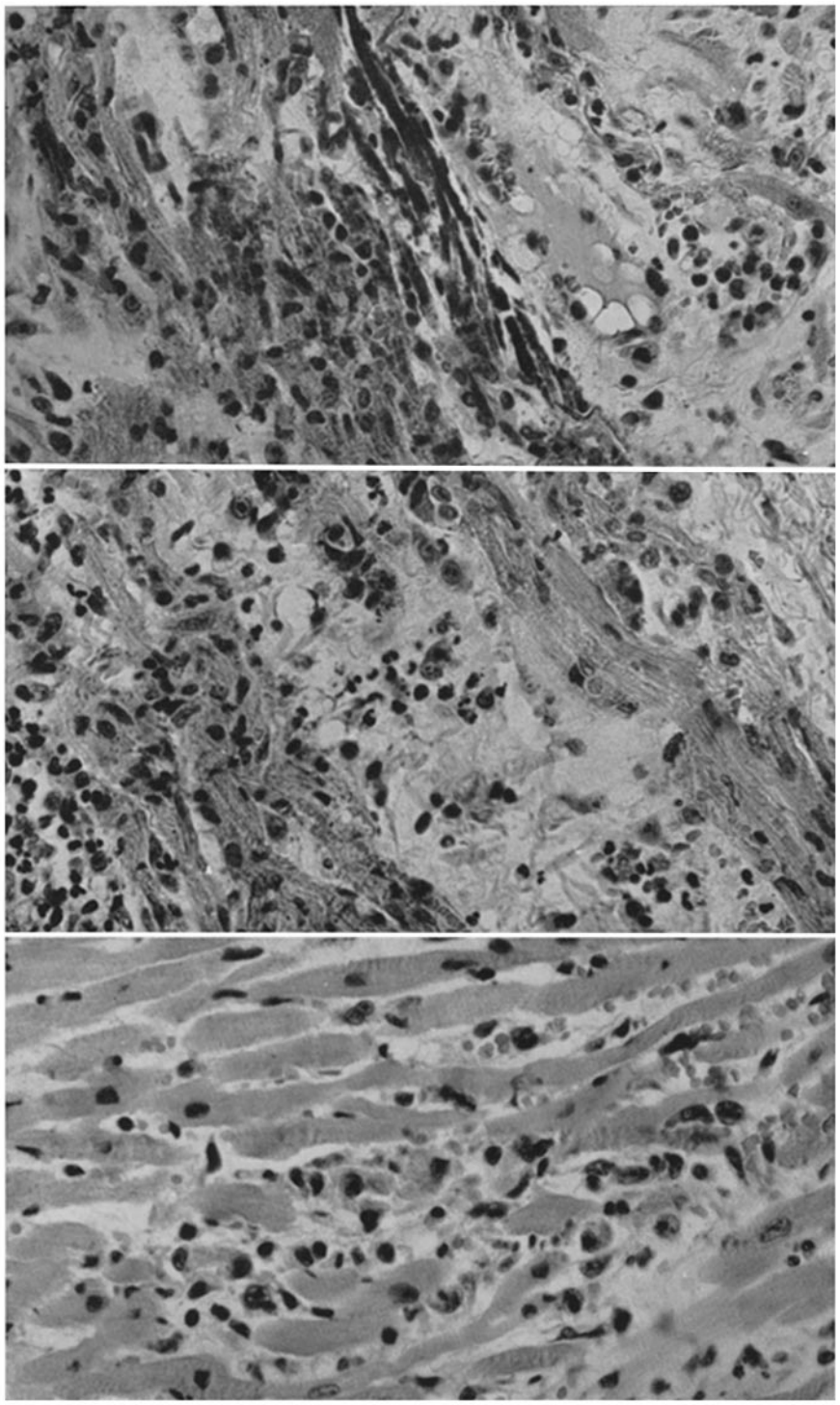


FIG. 5. The localized Schwartzman reaction provoked by an intravenous injection of 2 mg of solubilized mucopeptide into a rabbit pretreated 24 hr earlier with an intradermal injection of 100 μg of *E. coli* endotoxin. Endotoxin injected into the skin at the right; saline injected to the left.



6a

6b

6c

FIG. 6. *a*, *b*, *c*. Representative microscopic sections of the right ventricle of the heart of a rabbit 2 days after the intravenous injection of 2 mg of solubilized mucopeptide. Hematoxylin and eosin stain × 250. Myocardial cells are in various stages of degeneration and necrosis. A number of polymorphonuclear leukocytes have migrated into the tissue.