IMMUNOCHEMICAL STUDIES ON THE CROSS-REACTIVITY BETWEEN STREPTOCOCCAL AND STAPHYLOCOCCAL MUCOPEPTIDE*

BY WALTER W. KARAKAWA, PH.D., DIETMAR G. BRAUN,‡ M.D., HENRY LACKLAND, AND RICHARD M. KRAUSE, M.D.

(From the Department of Microbiology, Pennsylvania State University, University Park, Pennsylvania 16802, and The Rockefeller University, New York 10021)

(Received for publication 10 April 1968)

Previous studies on the immunology of streptococcal mucopeptide[1] indicate that the peptide moiety isolated by enzymatic means is a dominant antigenic determinant (1). More recently, it has also been shown that the hexosamine polymer may be antigenic (2). In an extension of these observations, immunologic cross-reactions between streptococcal and staphylococcal mucopeptides were noted, but the immunochemical basis for this cross-reactivity was not defined (3).

The mucopeptides of streptococci and staphylococci consist of repeating units of *N*-acetylglucosamine and *N*-acetyl muramic acid. In each case, tetrapeptides, composed of L-alanine, D-glutamic acid, lysine, 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 cross-linked to form a matrix (4, 5, 6). The cross-link extends from the carboxyl group of D-alanine of one tetrapeptide to the free amino group of the lysine of another tetrapeptide. The major chemical difference between the mucopeptides of streptococci and staphylococci is the composition of the peptide bridge spanning the tetrapeptides. The peptide bridge of hemolytic streptococci consists of L-alanyl-L-alanine (7), whereas that of staphylococci is a peptide of glycine (4).

Other studies have now directed attention to the antigenic properties of the mucopeptides of *Staphylococcus aureus*. Hisatsune et al. (8) isolated from a

* This study was supported by National Institutes of Health Grants No. AI 08015, AM AI 09846, and AI 08429; a Grant-in-Aid from the American Heart Association; it was conducted in part under the sponsorship of The Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiological Board, and supported by the Office of the Surgeon General, Department of the Army, Washington, D. C.

‡ United States Public Health Service post-doctoral International Fellow.

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

culture filtrate of strain Wiley a serologically active peptide component which had an amino acid composition similar to the peptide moiety of mucopeptide. The studies described here indicate that the immunologic cross-reactivity between the streptococcal and staphylococcal mucopeptide is dependent in part on the similarity of their tetrapeptides.

Materials and Methods

Bacterial Strains.—Streptococcal strains were obtained from Dr. R. C. Lancefield, Rockefeller University, N. Y. Staphylococcal strains were from the Department of Microbiology, Pennsylvania State University, Pa.

Preparation of Cell Walls.—Cell walls were prepared by the method of Bleiweis et al. (9) from: Group A-variant streptococci, strain T27A Variant; and *S. aureus* strains PSU, S_{29}, S_{53}, and S_{54}.

Preparation of Mucopeptide.—Particulate streptococcal mucopeptide was obtained as an insoluble residue after extraction of cell walls by the hot formamide method of Fuller (10) as described by Krause and McCarty (11). Particulate staphylococcal mucopeptides were prepared by hot trichloroacetic acid (TCA) treatment of cell walls as described by Park and Hancock (12).

Ion Exchange Chromatography.—The amino acids and amino sugars of acid hydrolysates of mucopeptide were determined by ion exchange chromatography on a modified Beckman-Spinco 120 C amino acid analyzer (13). 1 mg samples of mucopeptide were hydrolyzed in 1 ml of 6 N HCl for 5 and 24 hr in an evacuated, deaerated, sealed glass tube. The hydrolysate was evaporated to dryness in a rotary evaporator at 40°C, and the residue was dissolved in 3 ml of the pH 2.2 buffer used with the amino acid analyzer. The hydrolysate was passed through a Millipore filter before addition to the columns. Analyses were performed on duplicate 5 and 24 hr hydrolysis samples. Amino sugar values obtained at 5 and 24 hr were extrapolated to zero time. Complete release of amino acids was observed at 24 hr. During this time, under the conditions of hydrolysis employed here, there was no evident degradation of the major amino acids present in the mucopeptide.Therefore, 24 hr hydrolysis values were used to calculate the amino acids. Conversion factors for both amino acids and amino sugars were determined from standard mixtures which were analyzed at intervals between analyses of samples.

Total nitrogen, ash, and moisture of the mucopeptide were performed by Mr. Bella, analytical chemist, Rockefeller University.

Cell Wall Lytic Enzymes.—Partially purified AL-proteinase from a culture of *Myxobacter* (strain AL-1) was kindly supplied by Dr. R. S. Wolfe, Department of Microbiology, University of Illinois. Lysostaphin preparations were supplied through the courtesy of Dr. P. A. Tavormina, Mead Johnson Research Center, Evansville, Ind.

Serological Methods.—The preparation of mucopeptide antiserum by rabbit immunization with Group A-variant streptococci, and methods for qualitative and quantitative precipitin tests have been previously described (1).

Mucopeptide which had been solubilized by ultrasonic treatment was employed in the precipitin tests. The mucopeptide, suspended in saline, was cooled by an ice bath during treatment for 15 min in a 20 kc sonic oscillator.

Microzone Electrophoresis.—Methods for Microzone electrophoresis of serum proteins and isolated immune globulins have been reported (14).

Preparative Zone Electrophoresis of Serum.—The supporting medium was 0.5% agarose in Veronal buffer 0.05 M, pH 8.6. Electrophoresis was carried out for 24 hr at 4°C, 200 v, and 50 ma. The Agarose block was 25 cm long, 12.5 cm wide, and 0.5 cm deep.

Protein Determination.—Total protein in antiserum, serum fractions, and isolated mucopeptide antibody was determined by the biuret method (15).

RESULTS

Previous studies have directed attention to the immunologic cross-reactivity among the mucopeptides of several different Gram-positive bacteria, including staphylococci and hemolytic streptococci (3). Such an immunologic relation-

TABLE I

Chemical Compositions of Streptococcal and Staphylococcal Mucopeptide Preparations

Component	Streptococcal mucopeptide		Staphylococcal mucopeptide	
	μmole/mg	*μg/mg*	*μmole/mg*	*μg/mg*
Glucosamine	0.728	160.97	0.650	143.78
Muramic acid	0.622	182.48	0.604	177.09
Alanine	2.628	234.17	1.576	140.41
Glutamic acid	0.663	97.62	0.767	112.89
Lysine	0.717	104.84	0.709	103.68
Glycine	0.072	5.38	2.691	202.02
Histidine	0.006	0.98	0.021	3.30
Arginine	0.014	2.38	0.017	2.89
Aspartic acid	0.025	3.36	0.104	13.86
Threonine	0.013	1.51	0.067	8.04
Serine	0.010	1.00	0.101	10.57
Proline	<0.005		<0.005	
Valine	0.043	5.06	0.102	11.92
Methionine	0.015	2.20	0.039	5.83
Isoleucine	0.033	4.29	0.101	13.20
Leucine	0.047	6.22	0.120	15.69
Tyrosine	0.032	5.74	0.006	1.08
Phenylalanine	0.045	7.49	0.059	9.78
Ammonia		14.18		6.87
Total recovery		839.87		982.9

All values are given in terms of the anhydrous ash-free mucopeptide. The lyophilized staphylococcal mucopeptide was 7.29% moisture and 8.25% ash. The lyophilized streptococcal mucopeptide was 3.14% moisture and 2.04% 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.

ship is consistent with the observation that the mucopeptides of these two bacteria have a similar chemical structure. Presented in Table I are the chemical compositions of the mucopeptides of Group A–variant streptococci and *S. aureus*, strain PSU. The mucopeptides isolated from both organisms possess the same major amino acids although there is a difference in their mole ratios. These analytical data are in essential agreement with those previously reported (1, 4, 11, 16), with the exception that the glycine in the staphylococcal mucopep-

tide is somewhat less than expected. It is conceivable that this variation is due to the procedures employed to isolate this substance, or due to variation from strain to strain. Nevertheless, the analytical data are consistent with the notion that both streptococcal and staphylococcal mucopeptides contain tetrapeptides of L-alanine, D-glutamic acid, lysine, and D-alanine with a mole ratio of 1:1:1:1, which in the case of streptococci are cross-linked by L-alanyl-L-alanine, and in the case of staphylococci by a peptide of glycine. The following studies on the immunology of streptococcal and staphylococcal mucopeptides

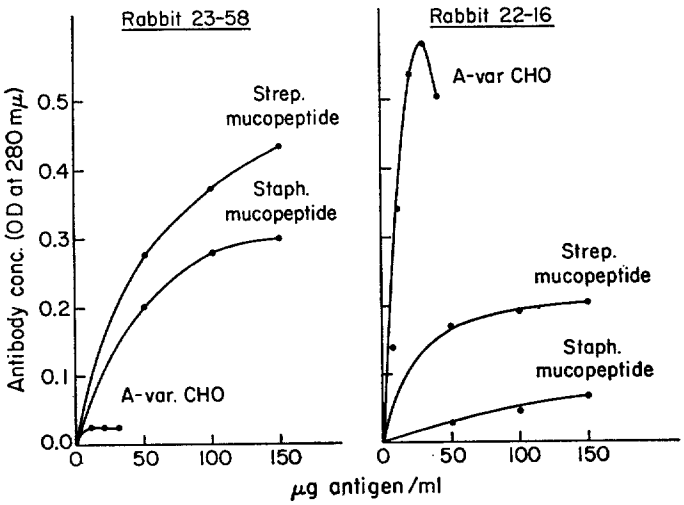

FIG. 1. Quantitative precipitin reaction between sonically disrupted mucopeptide of Group A-variant streptococci and *S. aureus* and Group A-variant carbohydrate, and Group A-variant antisera.

suggest that the antigenic cross-reactivity between these two substances is due to their similar tetrapeptides. In these experiments, the reaction between mucopeptide antibodies in Group A-variant antisera and staphylococcal mucopeptide have been examined in detail.

Cross-Reaction between Streptococcal and Staphylococcal Mucopeptides.—Although mucopeptide antibodies are produced by rabbits which are immunized with particulate mucopeptide which has been isolated from the cell wall, the antibody response is not as marked as that achieved when rabbits are immunized intravenously with vaccines prepared with intact Group A-variant streptococci, strain 486. However, such Group A-variant antisera exhibit great variability in the relative amount of antibodies to mucopeptide and to Group A-variant carbohydrate. Illustrated in Fig. 1 are the results of the quantitative precipitin tests which employed Group A-variant antisera, and streptococcal

and staphylococcal mucopeptides and Group A–variant carbohydrate. It should be noted that both streptococcal and staphylococcal mucopeptides solubilized by ultrasonic treatment gave strong reactions with antiserum R2358, whereas, the Group A–variant carbohydrate gave a minimal reaction. The situation was reversed in the case of antiserum R2216. This antiserum had a much higher concentration of Group A–variant antibodies than mucopeptide antibodies. This reciprocal relationship argues against the possibility that the mucopeptide precipitin reaction is due to incomplete extraction of cell wall

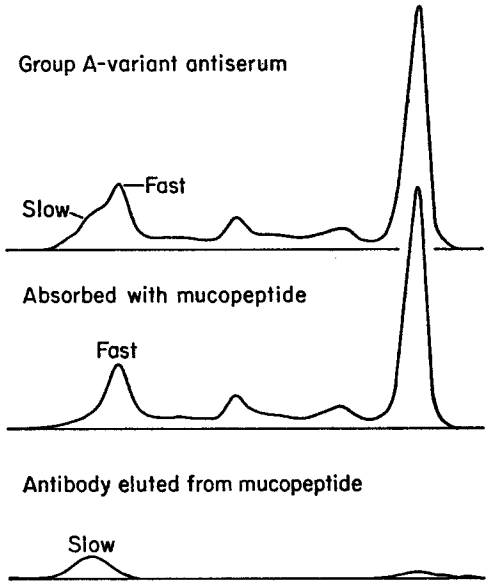

FIG. 2. Densitometric scans of Microzone electrophoretic patterns of Group A–variant antiserum R2358 before and after absorption with mucopeptide. Depicted in the bottom frame is the pattern of the γG-globulin eluted from the mucopeptide.

antigens from the mucopeptide, and supports the view that immunization with Group A–variant vaccines gives rise to antibodies with A–variant specificity and antibodies with mucopeptide specificity.

Isolation of Mucopeptide Antibodies from Antiserum.—Mucopeptide antibodies were isolated from Group A–variant antiserum by absorption onto particulate staphylococcal mucopeptide followed by elution at acid pH.

1 mg of staphylococcal mucopeptide was added to each milliliter of antiserum. After storage for 24 hr at 4°C, the particulate mucopeptide was collected by centrifugation and was washed three times with cold saline. The antibody was dissociated from the particulate mucopeptide by the addition of saline adjusted to pH 2 with HCl. After centrifugation, the supernatant containing the antibody was neutralized with 0.1 M Tris buffer [tris(hydroxymethyl)aminomethane], pH 8.0, in 0.5 M NaCl.

Electrophoretic patterns of unabsorbed serum, serum absorbed with mucopeptide, and antibodies eluted from the mucopeptide are depicted in Fig. 2. The major portion of the slow γ-globulin component was absorbed by the mucopeptide. The γ-globulin recovered from the mucopeptide had a migration similar to this slow component. The isolated antibody was identified as γG-globulin by means of immunoelectrophoresis. The antibody eluted from the mucopeptide did not react with Group A–variant carbohydrate. The antibodies to Group A–variant carbohydrate were identified in the fast γ-globulin peak.

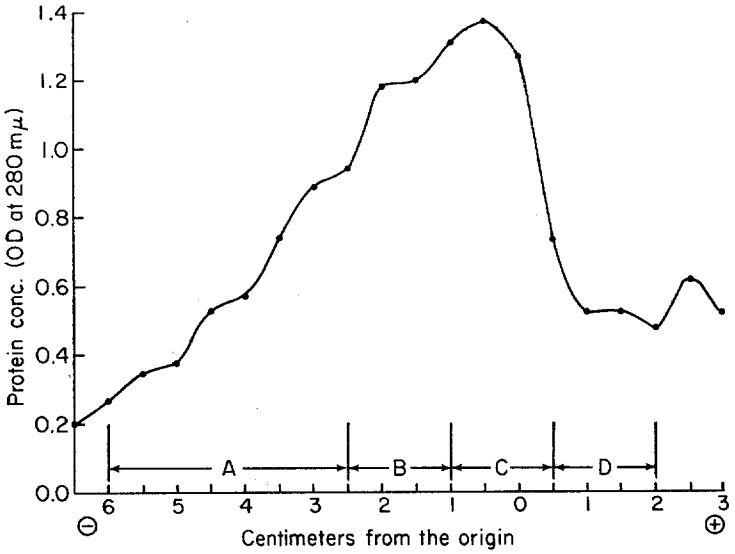

FIG. 3. Preparative zone electrophoresis of Group A–Variant antiserum R2358. The protein eluted from 0.5 cm segments of the Agarose block was measured at 280 mμ. Only protein values are recorded for segments in the γ-globulin region of the block. The protein fractions eluted from the segments were combined into 4 pools (A, B, C, D) designated in the figure.

Because these absorption studies of this Group A–variant antiserum with particulate mucopeptide indicate that the antibodies to mucopeptide have a slower electrophoretic migration than the Group A–variant antibodies, it has proved feasible to separate these antibodies from each other by means of preparative zone electrophoresis.

The protein eluted from 0.5 cm segments of an Agarose block is presented in Fig. 3. Eluates were pooled as indicated in the figure, and the pools designated A, B, C, and D. The pools were concentrated and dialyzed against 0.1 M Tris buffer, pH 8.0, in 0.5 M NaCl. Depicted in Fig. 4 are Microzone electrophoretic patterns of these pools and the original antiserum.

Group A–variant antibodies were detected only in pool D. Pools A and B contained the bulk of the antibodies to mucopeptide. This is illustrated in the

quantitative precipitin curves in Fig. 5. The volume of each pool was adjusted by the addition of saline so that the protein content was 8 mg/ml. Pools C and D were appreciably less reactive with staphylococcal mucopeptide than pools A and B.

Isolation of Haptenic Inhibitors from an Enzymatic Digest of Mucopeptide.— The peptide moiety has been identified as an immunodominant group of streptococcal mucopeptide (1) and staphylococcal mucopeptide (8). Furthermore, a common feature between the peptide moieties of both streptococcal and staphylococcal mucopeptides is a tetrapeptide. It is reasonable to assume

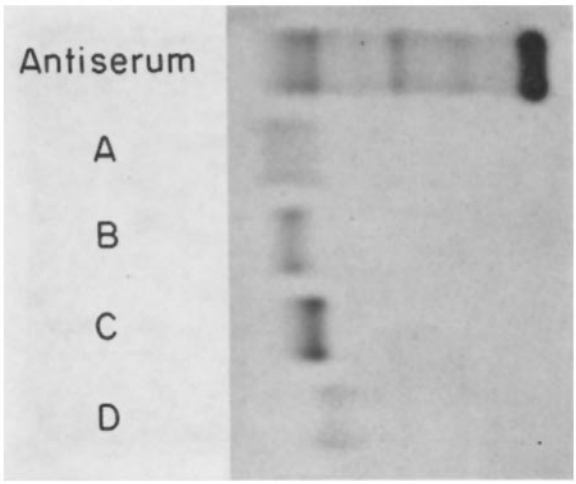

FIG. 4. Microzone electrophoresis of Group A–variant antiserum R2358 and γ-globulin pools A, B, C, and D recovered from preparative electrophoresis of the serum as depicted in Fig. 3.

therefore, that the immunologic cross-reactivity of streptococcal and staphylococcal mucopeptides is dependent upon this common tetrapeptide. Evidence in support of this view was obtained by demonstrating that a peptide moiety of staphylococcal mucopeptide, isolated by enzymatic means, inhibited the precipitin reaction between staphylococcal mucopeptides and antibodies to streptococcal mucopeptide.

Various methods were employed to isolate and identify haptenic fractions of staphylococcal mucopeptide which had been digested with muralytic enzymes. These methods, which include ion exchange chromatography and Sephadex gel filtration, have been employed previously to isolate the haptenic peptide moiety of streptococcal mucopeptide (1).

The two lytic enzyme preparations which were employed to digest the staphylococcal mucopeptide were AL-proteinase, derived from *Myxobacter* strain AL-1, and lysostaphin. Recent studies have shown that AL-proteinase

preparations contain activities which hydrolyze *N*-acetyl muramyl-L-alanine, D-alanyl-glycine, and glycyl-glycine (17). The lysostaphin preparation has the enzyme specificities similar to AL-proteinase, with the exception that it lacks the capacity to split D-alanyl-glycine (18, 19). But, in addition lysostaphin hydrolyses the glycosidic bonds in the hexosamine polymer.

100 mg of staphylococcal mucopeptide was suspended in 25 ml of Tris buffer, pH 9. 10 mg of AL-proteinase were added to the suspension and the mixture incubated at 37°C for 24 hr according to the method of Ensign and Wolfe (20). The digest was dialysed in cellophane tubing against frequent changes of distilled water, and the dialysates were pooled, concentrated,

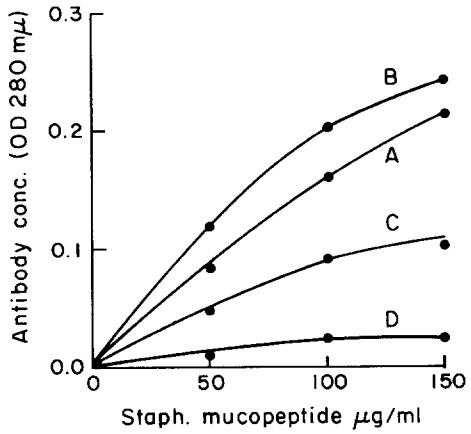

FIG. 5. Quantitative precipitin reaction between sonically disrupted staphylococcal mucopeptide and the γ-globulin pools A, B, C, and D recovered from preparative electrophoresis of serum R2358 as depicted in Fig. 3. The protein content of each pool was adjusted to 8 mg/ml and 0.1 ml was employed in the precipitin tests.

and lyophilized. The lyophilized product was resuspended in 0.02 M $(NH_4)_2CO_3$, pH 8.6, and separated on a DEAE-cellulose column equilibrated and eluted with this buffer. Three fractions, designated I, II, and III, were collected from the DEAE-cellulose column as depicted in Fig. 6 *a*. Fraction I was only present in trace amounts and was discarded. Fractions II and III inhibited the mucopeptide precipitin reaction. Fraction II was further resolved by G15-Sephadex gel filtration as previously described (1). A fall-through fraction strongly inhibited the mucopeptide precipitin reaction. A second fraction, which was retarded by the column, was much less inhibitory. The fall-through fraction was labeled IIa and the retarded fraction IIb. They were lyophilized to remove carbonate buffer and saved for subsequent chemical and immunologic analysis.

Similar procedures were employed to isolate haptenic fractions from staphylococcal mucopeptide which was digested with lysostaphin by the method of Schindler and Schuhardt (18). The dialyzable material from 100 mg of a lysostaphin digest of staphyloccal mucopeptide was fractionated on a DEAE-cellulose column equilibrated and eluted with 0.02 M $(NH_4)_2CO_3$ buffer, pH 8.6. In this case, as depicted in Fig. 6 *b*, three major fractions designated I, II, and III were obtained. Fraction I, which represented the fall-through peak, possessed much less inhibitory activity than fraction II and III. Preliminary chemical analysis indicated that

fraction I was rich in hexosamines and fraction II was composed almost entirely of amino acids. Because fraction II contained both hexosamines and amino acids further resolution was not attempted. Fraction I was the source material for isolating the hexosamine moiety and fraction III the source material for isolating a haptenic peptide. These fractions were further purified by G 15-Sephadex gel filtration as described above. In both cases, only the fall-through material was collected for subsequent chemical and serologic analysis. The lyophilized products were labeled fraction I and fraction III.

Presented in Table II are the chemical analyses of the purified haptenic fractions isolated by DEAE-cellulose chromatography from the AL-proteinase

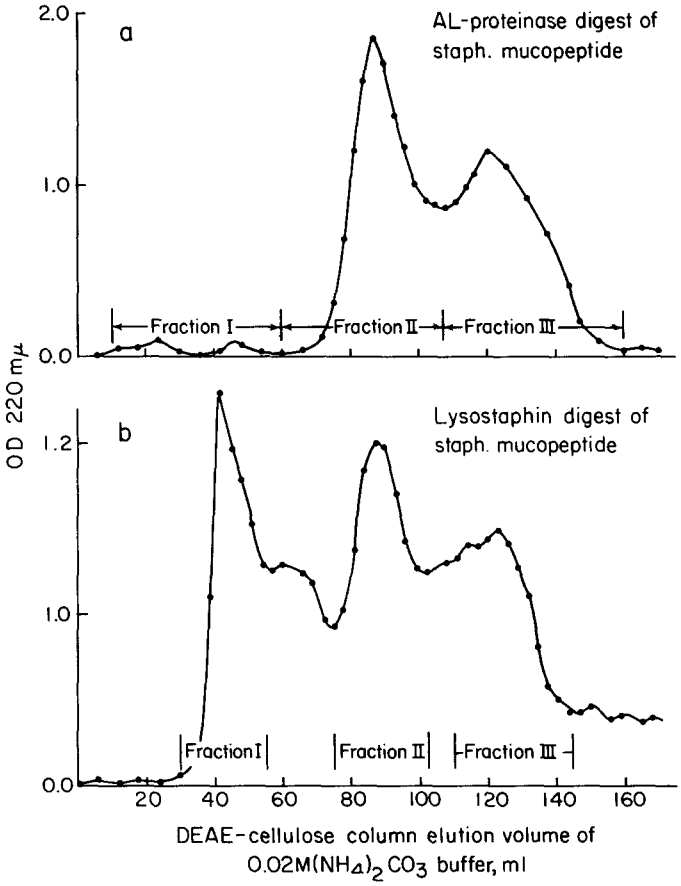

FIG. 6 a. DEAE-cellulose column chromatography of the dialyzable material of the AL-proteinase digest of staphylococcal mucopeptide. Column dimensions 300 × 25 mm. Buffer: 0.02 M $(NH_4)_2CO_3$, pH 8.6 at 4°C. Eluted material was pooled as designated in the figure into fractions I, II, and III.

FIG. 6 *b*. DEAE-cellulose chromatography performed as described above in the dialyzable material of a lysostaphin digest of staphylococcal mucopeptide. Eluted material was pooled as designated in the figure into fractions I, II, and III.

digest and the lysostaphin digest of staphylococcal mucopeptide. The analysis of mucopeptide has been included for comparison. The AL-proteinase digest yielded a glycine-rich fraction as well as a peptide fraction. Fraction IIa contained the amino acids of the tetrapeptide and a portion of the polyglycine bridge. Fraction IIb was predominantly glycine, presumably derived from the polyglycine bridge. Similar split products have been isolated by others (17, 19). The components of interest isolated from the lysostaphin digest included a hexosamine-rich fraction and a peptide moiety. Fraction I was primarily derived

TABLE II

Chemical Compositions of Dialyzable Fractions of Staphylococcal Mucopeptide Released by Either AL-Proteinase or Lysostaphin

Components	Mucopeptide	AL-proteinase extract		Lysostaphin extract	
		Fraction IIa	Fraction IIb	Fraction I	Fraction III
μmole/mg					
Glucosamine	0.650	*	*	1.765	*
Muramic acid	0.604	*	*	1.821	*
Alanine	1.576	1.635	0.380	0.216	2.309
Glutamic acid	0.767	1.176	0.226	0.090	1.214
Lysine	0.709	1.035	0.163	0.106	1.303
Glycine	2.691	4.967	7.706	0.232	3.260
Recovery, %		84.3	66.9	97.2	82.0

*, trace amount.

For the sake of brevity, all amino acid values for enzyme digest fractions which were 0.1 μmole/mg or less are not presented in the table. The values are calculated on the basis of the lyophilized weight of the sample, and not on the ash-free dry weight.

from the hexosamine polymer. Fraction III is similar to the peptide fraction IIa isolated from the AL-proteinase digest. The recovery of dialyzable hexosamine material in fraction I is due to the lysozyme-like activity of lysostaphin. It is to be noted, however, that no such material was liberated from mucopeptide by the action of AL-proteinase.

Illustrated in Fig. 7 are the results of inhibition of the mucopeptide precipitin reaction with the fractions isolated from the enzymatic digests of staphylococcal mucopeptide. The reactions were carried out at antigen-antibody equivalence. 4 mg/ml of the peptide moiety from the lysostaphin digest fraction III inhibited 62% of the reaction between staphylococcal mucopeptide and streptococcal antiserum. Although not shown in Fig. 7, it should be mentioned parenthetically that the peptide moiety from AL-proteinase fraction IIa was an equally good inhibitor. On the other hand, the glycine-rich moiety (AL-proteinase fraction IIb) and the hexosamine-rich moiety (lysostaphin digest fraction I) were less

effective inhibitors. The inhibition achieved with the hexosamine-rich fraction does merit additional comment, however. This suggests that the antiserum also contains antibodies directed against the hexosamine polymer of the mucopeptide. Such a finding is consistent with results reported earlier (2). While experience with these inhibition studies is still limited, the picture which is emerging is that the peptide moiety is the primary determinant of mucopeptide, and the hexosamine polymer is a secondary determinant.

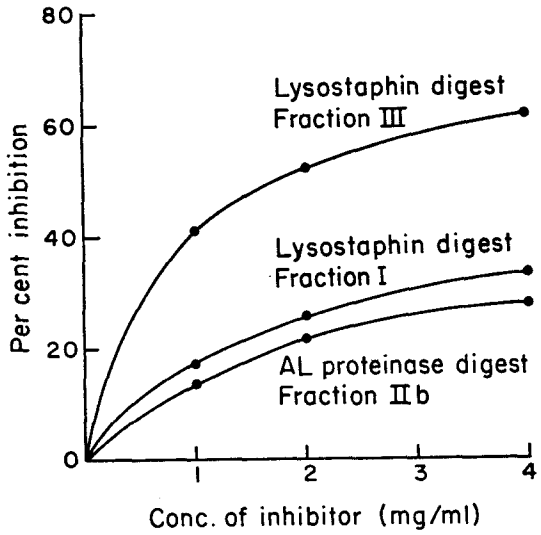

FIG. 7. Inhibition of the quantitative mucopeptide precipitin reaction with: hexosamine-rich fraction I, and peptide-moiety fraction III isolated from the lysostaphin digest of mucopeptide; and glycine-rich peptide fraction IIb isolated from AL-proteinase digest of mucopeptide. Recovery of these fractions is depicted in Fig. 6.

Further evidence for the immunologic cross-reactivity of the mucopeptides of streptococci and staphylococci was obtained in studies which employed the peptide isolated from a *Streptomyces albus* enzymes digest of streptococcal mucopeptide (1). In a final volume of 1 ml, 4 mg were added to a precipitin reaction consisting of 150 μg of solubilized staphylococcal mucopeptide and 0.1 ml of streptococcal antiserum. In comparison with the control, precipitation was inhibited by 62%.

One possible interpretation of these results is that the streptococcal antiserum contains one population of antibodies which reacts with the streptococcal mucopeptide and another which reacts with staphylococcal mucopeptide. In a final series of experiments, it was shown that the serum contained antibody which reacted with both mucopeptides. The antibody employed in these experiments

had been recovered from the antiserum by absorption onto particulate streptococcal mucopeptide and subsequently recovered as described in an earlier section. Such antibody reacted with the heterologous staphylococcal mucopeptide. Furthermore, the reaction was inhibited by 65% with the peptide (fraction III) isolated from the lysostaphin digest of staphylococcal mucopeptide.

It did not prove feasible by enzymatic means to isolate from mucopeptide a tetrapeptide which was devoid of glycine. Nevertheless, the capacity of the peptide moiety to inhibit streptococcal and staphylococcal mucopeptide precipitin reactions is probably dependent upon the tetrapeptide portion. This is supported by the fact that only the tetrapeptide and not the glycine bridge is common to both streptococcal and staphylococcal mucopeptide.

TABLE III

Inhibition of Staphylococcal Mucopeptide Precipitin Reaction by Synthetic Peptides

Inhibitors	Inhibition
10 mg/ml	%
Glycyl-L-glutamic acid	12.4
DL-alanine	3.4
L-alanyl-L-alanine	10.0
Poly DL-alanine	9.7
L-alanyl-L-lysine	10.0
L-alanyl-glycyl-glycine	12.4
L-alanyl-L-alanyl-L-alanine	18.4
L-alanyl-L-glutamic acid	34.4

All precipitin reactions were carried out with 0.1 ml of antiserum and 150 μg of solubilized mucopeptide in a final volume of 1 ml. Per cent inhibition values were obtained by comparison of the amount of antibody recovered in the inhibited reactions to that recovered in a non-inhibited reaction.

Because the peptide moiety was an effective inhibitor of the mucopeptide precipitin reaction, synthetic peptides were employed in a similar fashion. The results are presented in Table III. Inhibition was most prominent with L-alanyl-L-glutamic acid. These data are in agreement with the view that the tetrapeptide of mucopeptide is an antigenic determinant.

DISCUSSION

Recent evidence from a number of sources indicates that the bacterial mucopeptides are potentially antigenic (1, 3, 8, 21, 22). This is not, however, a wholly unexpected development. Basically, mucopeptide consists of a hexosamine polymer with peptide side chains linked to the polymer through the carboxyl group of the muramic acid residues. Each of these two major components is a potentially immunodominant group. Previous studies have shown that anti-

bodies to both the hexosamine polymer and the peptide moiety may be detected in the sera of rabbits after immunization with Group A–variant streptococci (1, 2).

It is, at present, a mystery why rabbits immunized with Group A–variant streptococci (23) are more likely to produce mucopeptide antibodies than rabbits immunized with Groups A or C streptococci (24). One possible explanation may stem from antigenic competition between the group-specific carbohydrate and the mucopeptide. The majority of rabbits immunized with Groups A and C streptococci usually produce 3—15 mg/ml of antibodies to group carbohydrate. In some cases, over 80% of the total γ-globulin in immune sera may be antibody to the carbohydrate antigen. On the other hand, the Group A–variant carbohydrate appears to be a poor antigen. Many Group A–variant antisera contain less than 1 mg/ml of group-specific antibody. In the face of a feeble response to carbohydrate antigen, the mucopeptide may initiate a more vigorous immune response.

Variability in the immune response to mucopeptide may be in part genetically determined. Genetic factors which control the immune response to synthetic antigens have now been well established in the case of mice and guinea pigs. Pinchuck and Maurer have shown that the ability of mice to form antibodies against the random terpolymer $glu_{57}lys_{38}ala_5$ is controlled by a codominant Mendelian factor (25). In guinea pigs, it has been shown that the ability of poly L-lysine to act as a hapten carrier for the 2,4-dinitrophenyl (DNP) group is transmitted as a simple Mendelian determinant (26, 27, 28, 29). Breeding studies with rabbits currently underway may identify genetic factors which control a vigorous immune response to mucopeptide.

In the studies reported here, the immunologic cross-reactivity between streptococcal and staphylococcal mucopeptide has been studied in detail. The evidence suggests that this cross-reactivity is dependent in large part on the fact that a chemically similar tetrapeptide is a common antigenic determinant of both mucopeptides. It should be stressed, however, that the hexosamine polymer is also a common feature of both mucopeptides. Presumably cross-reactivity can depend secondarily upon this common hexosamine polymer. Precipitin inhibition was achieved by a dialyzable hexosamine-rich moiety isolated from a lysostaphin digest of staphylococcal mucopeptide. Such a result is indicative that the hexosamine polymer of staphylococcal mucopeptide has antigenic properties similar to the hexosamine polymer of streptococcal mucopeptide (2).

The antigenicity of the tetrapeptide of mucopeptide is reminiscent of the studies of Fuchs and Sela on the locus of the immunologically important area of a multichained polymer of amino acids (30). For example, a polymer which was nonantigenic remained so after the addition of peptide side chains consisting of only a single amino acid. On the other hand, synthetic multichained poly-

alanine was converted to an immunogenic substance if the side chain peptides contained glu-lys, glu-phe, glu-his, or glu-leu.

In view of the similarity in chemical composition and structure among the bacterial mucopeptides, it will not be surprising if future studies reveal widespread immunologic cross-reactivity. Furthermore, the occurrence of mucopeptides in all bacteria insures prolonged and continuous exposure of animals and man to these substances and provides a means for the natural acquisition of sensitivity to mucopeptide. It is conceivable that the immune response to this family of polymers may play a role in bacterial allergy. Under these circumstances the biological significance of mucopeptide immunity merits attention.

At least two different laboratories have now quite independently directed attention to the toxic properties of mucopeptide. Rotta and coworkers have shown that the streptococcal cell wall mucopeptide will induce fever in rabbits, enhance nonspecific resistance in mice against subsequent challenge with streptococci, and prepare and provoke the localized Shwartzman phenomenon (31).[2] Abdulla and Schwab have observed dermal necrosis following injection of mucopeptide into the skin of rabbits (32). Schwab and coworkers have produced intermittent subcutaneous nodular lesions after injections of streptococcal cell wall particles into the skin of rabbits (33). It is their view that mucopeptide may be an essential component of the cell wall particles which induce these lesions (32). These various biological properties of mucopeptide may be dependent upon its inherent toxicity, but it is also possible that these toxic reactions to mucopeptide after injection by various routes are manifestations of previously acquired immunity to mucopeptide.

SUMMARY

Particulate mucopeptides of Group A–variant streptococci and *Staphylococcus aureus*, solubilized by ultrasonic treatment, give a precipitin reaction with the sera of rabbits immunized with Group A–variant streptococci. γ-G globulin antibodies have been recovered from these sera which react with the mucopeptides but not with the Group A–variant carbohydrate.

The immunochemical basis for the cross-reactivity between the streptococcal and staphylococcal mucopeptides was investigated in detail. Three chemically different fractions have been isolated from enzymatic digests of staphylococcal mucopeptide and were employed as haptenic inhibitors of the precipitin reaction. A fraction consisting of the peptide moiety of mucopeptide was the strongest inhibitor, whereas the hexosamine-rich fraction was less effective. The third fraction, rich in glycine, was least effective.

It is suggested that the immunologic cross-reactivity between streptococcal

[2] Rotta, J., and B. Bednar. Personal communication.

and staphylococcal mucopeptide is due to the fact that these two substances contain chemically similar tetrapeptides. The hexosamine polymer which is identical for both mucopeptides may also contribute to their cross-reactivity.

BIBLIOGRAPHY

1. Karakawa, W. W., and R. M. Krause. 1966. Studies on the immunochemistry of streptococcal mucopeptide. *J. Exptl. Med.* **124**:155.
2. Karakawa, W. W., H. Lackland, and R. M. Krause. 1967. Antigenic properties of the hexosamine polymer of streptococcal mucopeptide. *J. Immunol.* **99**:1178.
3. Karakawa, W. W., H. Lackland, and R. M. Krause. 1966. An immunochemical analysis of bacterial mucopeptides. *J. Immunol.* **97**:797.
4. Salton, M. R. J. 1964. The Bacterial Cell Wall. Elsevier Publishing Co., Amsterdam. 133.
5. Perkins, H. R. 1963. Chemical structure and biosynthesis of bacterial cell walls. *Bacteriol. Rev.* **27**:18.
6. 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. Williams and Wilkins Co., Baltimore. 52.
7. Petit, J. F., E. Munoz, and J. M. Ghuysen. 1966. Peptide cross-links in bacterial cell wall peptidoglycan studied with specific endopeptides from *Streptomyces albus*. *Biochemistry.* **5**:2764.
8. 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.
9. Bleiweis, A. S., W. W. Karakawa, and R. M. Krause. 1964. Improved techniques for the preparation of streptococcal cell walls. *J. Bacteriol.* **88**:1198.
10. Fuller, A. T. 1938. The formamide method for the extraction of polysaccharides from haemolytic streptococci. *Brit. J. Exptl. Pathol.* **19**:130.
11. 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 Group A and A-variant streptococci. *J. Exptl. Med.* **114**:127.
12. Park, J. T., and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell wall mucopeptide and other polymers in cells of *Staphylococcus aureus*. *J. Gen. Microbiol.* **22**:249.
13. 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.
14. Osterland, C. K., E. J. Miller, W. W. Karakawa, and R. M. Krause. 1966. Characteristics of streptococcal group specific antibody isolated from hyperimmune rabbits. *J. Exptl. Med.* **123**:599.
15. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**:751.
16. Heymann, H., L. D. Zeleznick, and J. A. Manniello. 1961. On the mucopeptide fraction of streptococcal cell walls. *J. Am. Chem. Soc.* **83**:4859.
17. Tipper, D. J., J. L. Strominger, and J. C. Ensign. 1967. Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. VII. Mode of action of the

bacteriolytic peptidase from *Myxobacter* and the isolation of intact cell wall polysaccharides. *Biochemistry* **6**:806.

18. Schindler, C. A., and V. T. Schuhardt. 1964. Lysostaphin: a new bacteriolytic agent for the staphylococcus. *Proc. Natl. Acad. Sci. U. S.* **51**:414.
19. Browder, H. P., W A. Zygmunt, J. R. Young, and P. A. Tavormina. 1965. Lysostaphin: enzymatic mode of action. *Biochem. Biophys. Res. Commun.* **19**:383.
20. Ensign, J. C., and R. S. Wolfe. 1965. Lysis of bacterial cell walls by an enzyme isolated from a myxobacter. *J. Bacteriol.* **90**:395.
21. Abdulla, E. M., and J. H. Schwab. 1965. Immunological properties of bacterial cell mucopeptides. *Proc. Soc. Exptl. Biol. Med.* **118**:359.
22. Gotschlich, E. C., and T. Y. Liu. 1967. Structural and immunological studies on the pneumococcal C polysaccharide. *J. Biol. Chem.* **242**:463.
23. McCarty, M. 1956. Variation in the group-specific carbohydrate of Group A streptococci. II. Studies on the chemical basis for serological specificity of carbohydrates. *J. Exptl. Med.* **104**:629.
24. McCarty, M. 1964. The streptococcal cell wall and its biological significance. In The Streptococcus, Rheumatic Fever, and Glomerulonephritis. J. W. Uhr, editor. Williams and Wilkins Co., Baltimore. 3.
25. Pinchuck, P., and P. H. Maurer. 1965. Antigenicity of polypeptides (poly alpha amino acids). XVI. Genetic control of immunogenicity of synthetic polypeptides in mice. *J. Exptl. Med.* **122**:673.
26. Kantor, F. X., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. I. Antigenicity of DNP-polylysine and DNP copolymer of lysine and glutamic acid in guinea pigs. *J. Exptl. Med.* **117**:55.
27. Benacerraf, B., A. Ojeda, and P. H. Maurer. 1963. Studies on artificial antigens. II. The antigenicity in guinea pigs of arsanilic acid conjugates of copolymers of D- or L-α-amino acids. *J. Exptl. Med.* **118**:945.
28. Levine, B. B., A. Ojeda, and B. Benacerraf. 1963. Studies on artificial antigens. III. The genetic control of the immune response to hapten-poly-L-lysine conjugates in guinea pigs. *J. Exptl. Med.* **118**:953.
29. Levine, B. B., and B. Benacerraf. 1965. Genetic control in guinea pigs of immune response to conjugates of haptens and poly-L-lysine. *Science.* **147**:517.
30. Fuchs, S., and M. Sela. 1964. Studies on the chemical basis of antigenicity of proteins. VII. Antigenicity of some new synthetic polypeptides and polypeptidyl gelatins. *Biochem. J.* **93**:566.
31. 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. Exptl. Med.* **122**:877.
32. 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.
33. Schwab, J. H., W. J. Cromartie, and B. S. Robertson. 1959. Identification of a toxic cellular component of Group A streptococci as a crude complex of group-specific C polysaccharide and a protein. *J. Exptl. Med.* **109**:43.