EXAMINATION OF THE L FORMS OF GROUP A STREPTOCOCCI FOR THE GROUP-SPECIFIC POLYSACCHARIDE AND M PROTEIN*‡

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Plasticity and osmotic and mechanical fragility have been recognized as properties common to L forms of bacteria and pleuropneumonia-like organisms since their earliest study (1–3). These characteristics have led to the conclusion that these microbial forms lack rigid cell walls. Electron micrographs have provided evidence of a difference in outer membrane structure. Smith, Hillier, and Mudd (4) observed a "veil-like" membrane in studies on an L form as compared to the thicker cell wall of the bacterium. In the studies reported here, we have found that the L form of the group A streptococcus lacks an antigen which is known to be a major constituent of the cell wall of the bacterial form.

Materials and Methods

Transformation from the streptococci to L forms was induced by penicillin on solid media containing horse serum and approximately 3.5 per cent NaCl (5). Subcultures of the L colonies were made on the same media containing more than 1000 units of penicillin per ml. until growth was sufficiently heavy to permit serial subculture in liquid media (6). Immunological and chemical studies were made on broth cultures of four strains of group A streptococci. Polysaccharide studies were conducted on two strains: GL8 and AED. M protein tests were carried out on three strains: GL8, type 19; D58, also known as the Richards strain, type 3; and ADA, type 14. The AED was found non-typable. The L form from which the "recovered" AED streptococcus was obtained was isolated from the coccus separately from the L form used for the immunological and chemical studies. This recovered coccus

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was obtained on the third consecutive subculture of its L form in the absence of penicillin, the L form having been transplanted three times previously in the presence of the antibiotic.

Extraction of antigens was done by the Lancefield hot acid method (7). Three lots of group A and one lot each of groups B and G and Types 3, 12, 14, and 19 antisera were used.[1]

Paper chromatographic examination for glucosamine was carried out by the two-dimensional method of Stoffyn and Jeanloz (8). Chromatograms for rhamnose were made by a one dimensional ascending method utilizing an ethyl acetate, glacial acetic acid, and water-solvent system. Quantitative methyl pentose determinations were made by the Dische and Shettles method (9).

TABLE I

Tests on Acid Extracts of L Forms and Bacteria

	Strain AED			Strain GL8	
	Coccus	L Form	Recovered coccus	Coccus	L form
Dry weight of cells extracted, *mg*.....	113	270	27.0	27.2	294
Final volume of extract, *ml*.....	18	12	22	25	38
Total methyl pentose of extracts, mg. rhamnose.....	6.23	0.029	1.32	2.125	0.000
Per cent of cells methyl pentose.....	5.51	0.011	4.89	7.81	0.000
Precipitin reactions* with anti-A serum.....	Positive in dilution 10^0 to 10^{-4}	Negative in dilution 10^0 to 10^{-4}	Positive	Positive	Negative in dilution 10^0 to 10^{-4}

* Control precipitin reactions performed on all extracts were negative with group B and G antisera in all dilutions.

RESULTS

Examination for Polysaccharide.—Hot acid extracts of small amounts of broth culture of the L form gave no reaction with group A antisera. Since extraction of similar amounts of streptococcal culture yielded sufficient polysaccharide to elicit strong reactions with the antisera, it was apparent that this antigen was either lacking in the L form or present in smaller amounts. These possibilities were examined by utilizing relatively large amounts of bacteria and L forms for preparing extracts.

[1] The bacterial strains used in this study were kindly supplied to us by Drs. A. T. Wilson, H. C. Anderson, and J. Seal. Original typing of the bacterial strains was either made known to us by those supplying the strains or was performed by Dr. M. McCarty or by the Communicable Disease Center, United States Public Health Service. One lot of grouping antiserum was provided by Dr. R. M. Cole, National Microbiological Institute, Bethesda, and another lot provided by the Communicable Disease Center, Chamblee, Georgia. The third lot was prepared in this laboratory.

L forms and streptococci were collected in the amounts shown in Table I. The organisms were extracted 6 times with N/5 HCl in a boiling water bath, digesting 10 minutes for each extraction. The hydrolysates of each organism were pooled, neutralized with NaOH, and partially purified by the addition of 4 volumes of 95 per cent ethanol. The alcohol-soluble material was concentrated to dryness in a vacuum still at a temperature not exceeding 37°C., and then dialyzed until chloride-free. The final volume was recorded.

These extracts were examined for methyl pentose and for the group specific polysaccharide. The results of these studies are shown in Table I. Precipitin tests for the group A substance in the L form were negative in all dilutions tested, whereas the streptococcal extracts gave positive reactions in relatively high dilutions when related to the original dry weight of the organisms. Similarly, the quantity of methyl pentose found in the extracts of the bacterial

TABLE II

Chromatography on Sugar and Hexosamine Fractions of Streptococcal L Forms

Sample		Rhamnose	Glucosamine
mg.			
28.8	AED-L_{51}*	Negative	—
25.0	AED-L_{102}	"	—
30.2	Broth media	"	—
1.0	AED-Stroptococcus	Positive	—
23.9	AED-L_{51}	—	Positive
24.9	Broth media	—	"

All samples dialyzed, lyophilized, hydrolyzed with 2 N HCl, and sugar fraction separated on dowex 50 columns.

* Numerical subscript to L designation signifies the number of subcultures in the L form since isolation from the bacterium.

forms was within the expected range, whereas the value obtained with the extract of one L form was entirely negative and the other was positive for an amount so small as not to exceed the variation inherent in the method.

In the next experiments, whole organisms were examined for the presence of glucosamine and rhamnose, the constituents of the group-specific polysaccharide (10, 11).

L forms and bacteria were collected by centrifugation and washed with saline. The organisms and one lot of uninoculated broth were dialyzed in the cold against multiple changes of distilled water and lyophilized. Weighed samples were hydrolyzed with 2 N HCl in sealed tubes at 100°C. Hydrolysis time was 2 hours for the samples examined for rhamnose and 15 hours for those examined for glucosamine. The hydrolyzed specimens were dried *in vacuo* over P_2O_5 and NaOH and passed over Dowex 50 columns (12). The entire residue of the appropriate eluates was then dried under streaming nitrogen and examined chromatographically.

The results of the chromatograms on these preparations from whole organisms are shown in Table II. Whereas 1 mg. of streptococcus gave a strong

spot for rhamnose, this sugar was not detectable in the hydrolysate of 28 mg. of L form. In contrast, a small amount of glucosamine was found in 23.9 mg. of L form. No attempt was made to determine whether the cellular component containing the glucosamine was a protein or a polysaccharide.

The results of these experiments indicate that these strains of L form of the group A streptococcus do not contain the group-specific polysaccharide. It is also evident that they do not possess a rhamnose-containing variant of this carbohydrate.

M Protein Studies.—Three strains of L forms derived from cocci which made M protein in the coccal form were examined for M protein. Two strains gave negative reactions. The hot acid extract of the L form from 200 ml. of broth culture of one strain, the GL8, reacted with the same typing antiserum as the bacterial strain from which it was derived, type 19. This strain's L form, when tested more than 1 year after original isolation from the bacterium and after 94 serial subcultures, continued to give a positive precipitin test with type-specific antiserum.

Since the antigenically reactive portion of the M protein molecule is known to be highly labile to proteolytic digestion (13) the extract of this L form was exposed to trypsin and reexamined for the precipitating antigen.

Precipitin-positive acid extracts of the L form and coccus of GL8 were divided into equal portions. pH of these extracts was approximately 7.6 as tested with phenol red. Crystalline trypsin in a final concentration of approximately 0.1 mg./ml. was added to one portion and a like volume of saline was added to the other. These tubes were incubated in a 37°C. water bath for 30 minutes, followed by heating in a boiling water bath for 5 minutes.

Trypsin completely destroyed the type-specific precipitating antigen in the extracts of this L form and the coccus whereas dilution and heating did not alter its ability to react. It appears possible therefore that the GL8 strain made M protein in the L form, although the evidence on this point is not sufficient to warrant a final conclusion.

DISCUSSION

Production of the group-specific carbohydrate is a stable property of the bacterial cell. A change in the ratio of its monosaccharide components with consequent alteration in its immunological specificity has been observed to occur in rare instances (14) during the course of animal passage (15) of group A streptococci but total loss of the polysaccharide has not been reported. The lack of this carbohydrate or a recognizable variant of the polysaccharide in the L form is a significant alteration in cellular composition and probably is the result of the transformation of the bacterium into this form. If this is correct, certain implications are of interest.

McCarty has established that the group A polysaccharide is a major constituent of the bacterial cell wall and constitutes from 50 to 70 per cent of that structure

(11). He demonstrated that the cell walls of group A streptococci are resistant to digestion with papain, pepsin, trypsin, chymotrypsin, and ribonuclease and not solubilized by phenol, glycine, urea, or detergents. Treatment with a filtrate of *Streptomyces albus* caused complete disintegration of this structure and he presented evidence that the substrate for this enzyme is the cell wall polysaccharide. Therefore, it is logical to conclude that the presence of the polysaccharide or one of its constituents is essential to the rigidity of the coccus and its absence in the L form accounts for the plasticity of that form of the organism.

In contrast to the polysaccharide, production of M protein by the bacterium is a variable property. The finding of this antigen in only one of three strains of L form probably further reflects that variability. M protein, like the polysaccharide, has been found to be associated with the cell wall of the bacterial organism, but its structural importance has not been established (16, 17). At least the antigenically reactive portion of the molecule, if not the entire protein, can be removed by enzymatic digestion from cell wall preparations without altering their structural integrity (16), and from living cells without altering their viability (13). Thus neither the presence nor absence of this constituent in the L form permits us to draw conclusions as to alteration in structure. However, the presence of this antigen is of importance in establishing that the L form did in fact derive from the bacterium and it is important to note that the strain which was found to make M protein was also one of those found to lack the carbohydrate.

Weibull has commented on the similarity between L forms and protoplasts (18). He has obtained protoplasts from *B. megatherium* by digestion of living cells with lysozyme (19). These units although differing from the bacterium in form, retain structural and osmotic integrity and maintain many metabolic functions for several hours. Since isolated cell walls of *B. megatherium* have been demonstrated to undergo dissolution on digestion with lysozyme (20) and the substrate for this enzyme is thought to be the polysaccharide component (21), the protoplast is considered to be the bacterial organism devoid of at least the cell wall polysaccharide if not the entire cell wall. The absence of the cell wall polysaccharide from the streptococcal L form therefore provides direct evidence of a structural resemblance to protoplasts. One major feature distinguishes the L form from the protoplast at the present time. L forms are principally characterized by their ability to reproduce and maintain growth indefinitely in distinctive morphological forms, whereas reproduction of protoplasts has not been proven, although some suggestive observations have been presented (22).

The mechanism for the loss of the polysaccharide in the process of transformation of the streptococcus to the L form has not been established. It is conceivable that the organism makes the polysaccharide but is either unable to incorporate it in the proper structure, or that the material is digested by an enzyme produced by the L form. Another possibility is that the L form of this organism is unable to make the polysaccharide. This might be a result of exposure to penicillin since one of the effects of this antibiotic on bacteria is the accumulation of the uridine diphosphate compounds (23) and it has been suggested that these compounds are related to polysaccharide synthesis

(24). If this were the only factor involved, one would expect that the L form would regularly return to the original bacterial form on withdrawing penicillin but this is not the case. Transformation back to the streptococcus is a rare event in terms of number of L organisms giving rise to one bacterial colony. The group A streptococcus has been recovered from its L form on a number of occasions but not after more than 12 subcultures in the L form. Since the production of the carbohydrate is a stable property of the bacterial cell and its absence a stable property of the L form another possibility is that the loss of this substance may be the result of an alteration in the genetic material or the hereditable loss of a cytoplasmic particulate essential to the production of the polysaccharide (25, 26).

SUMMARY

Two strains of L forms of group A streptococci were examined for group-specific polysaccharide and found to lack this substance. One of these was found to make a substance that had several properties in common with M protein. It is suggested that the absence of the cell wall polysaccharide is responsible for the lack of rigidity of the L form and that the L form of this species closely resembles protoplasts as prepared from other species.

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