Cell-Wall Deficiencies in L-forms of *Staphylococcus aureus*

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SUMMARY

Three strains of penicillin-induced and one strain of D-cycloserine-induced L-forms of *Staphylococcus aureus* were investigated for components of the normal bacterial cell wall. None of the 4 strains was found to contain more than 0.008 % of its dry weight as muramic acid, indicating the lack of mucopeptide. These forms also lacked the cell-wall ribitol teichoic acid as shown by the failure to yield any material containing ribitol. Neither muramic acid nor ribitol was detected as acid-soluble nucleotides in the L-forms, and teichoic acid was not released into the supernatant medium. Serological evidence for polysaccharide A was not found, but there was serological identity between extracts of membranes obtained from staphylococci and extracts of membranes isolated from the L-forms.

INTRODUCTION

Since the report of Dienes & Sharp (1956) on the isolation of various organisms in the L-phase by using a medium containing a high salt concentration, serum and penicillin, there have been several studies on the biology of the L-forms, including those of *Staphylococcus aureus* (Schönfeld, 1959, 1961; Marston, 1961*a, b*; Williams, 1963). Although these reports, particularly the last, gave indirect evidence, such as osmotic fragility, phage and antibiotic insensitivity, for the lack of cell-wall material, no direct evidence was presented. Only one report has appeared on cell-wall components of staphylococcal L-forms; Sharp (1963) found a very low level of muramic acid in an L-form from staphylococcus H.

The mucopeptide of the cell wall has been shown to be absent or greatly decreased in amount both in streptococcal L-forms (Panos, Barkulis & Hayashi, 1959) and stable *Proteus* L-forms (Kandler, Hund & Zehender, 1958; Morrison & Weibull, 1962), and in the doubtfully related *Mycoplasma* group (Plackett, 1959). Further evidence for a deficiency in cell-wall components from L-forms has been provided by serological techniques; for example, the L-forms of group A streptococci were found to lack group-specific polysaccharide (Sharp, Hijmans & Dienes, 1957). Similar results had earlier been obtained in the cases of L-forms of *Streptobacillus moniliformis* (Klieneberger, 1942) and *Proteus* (Dienes, Weinberger & Madoff, 1950), although these studies antedated much important work on cell-wall structures. The present paper reports some attempts to demonstrate cell-wall components in L-forms of *Staphylococcus aureus*.

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METHODS

Organisms and media. Three strains of staphylococcal L-form, 1444L, 6L and 42EL, previously described by Williams (1963), were used. A fourth strain, L Rogers, isolated from a penicillinase-producing staphylococcus (phage type 42E) by growth in the presence of D-cycloserine, was also used.

The solid and liquid media used for growth of the penicillin-induced L-forms were those described by Williams (1963). The media used for L Rogers were essentially the same except that more sodium chloride was added to give a final concentration of 4.5% (w/v) and the penicillin was replaced by D-cycloserine (Lilly) 500 µg./ml.

Broth cultures of the L-forms were harvested after 48 hr incubation at 37° by centrifugation at 27,000 g for 10 min. The pellet was washed once with ice-cold salt solution of the appropriate strength and then extracted by grinding for 5 min. with cold 10% (w/v) trichloroacetic acid (TCA). The residue was recovered by centrifugation and dialysed in the cold overnight against distilled water and lyophilized.

The parent staphylococci were grown in Difco brain heart infusion broth overnight at 37°, centrifuged, washed once with physiological saline, once with distilled water and either lyophilized or used directly for the preparation of cell walls.

Preparation of cell fractions. Cell walls of the parent staphylococci were isolated by the method of Salton & Horne (1951), by using a Mickle tissue disintegrator and no. 12 ballotini beads.

Staphylococcal cell membranes and membranes from the L-form strains were isolated essentially as described by Freimer (1963) for similar structures from group A streptococci and osmotically fragile protoplasts. The supernatant fluids remaining after removal of cell walls and membranes from suspensions of disrupted staphylococci (or membranes from suspensions of lysed L-forms) were centrifuged at 100,000 g for 1 hr and the resulting 'ribosomal' gel (Davison & Baddiley, 1963) was lyophilized.

Mucopeptide fractions from whole staphylococci were isolated by the method of Park & Hancock (1960) and by extraction with hot formamide (Fuller, 1938). Whole L-form was also extracted using these methods and the resulting residues lyophilized.

Attempts to isolate teichoic acids from staphylococci and L-forms were made by extraction with cold 10% (w/v) TCA (Armstrong *et al.* 1958).

Paper chromatography. Samples for the chromatographic detection of amino acids and amino sugars were hydrolysed in 4N-HCl at 100° for 16 hr in sealed tubes. The compounds were separated on Whatman no. 1 paper by an ascending two-dimensional technique using the solvents: (a) *n*-butanol+acetic acid+water (120+30+50, by vol.); (b) 2, 4/2, 5 lutidine+water (180+70, by vol.).

The dried papers were dipped in ninhydrin reagent (0.2% (w/v) in acetone) and heated for 5 min. at 90°. Sugar alcohols were released by hydrolysis in 2N-HCl at 100° for 8 hr and were separated by descending chromatography on Whatman no. 1 paper with solvent *n*-butanol+acetic acid+water (120+30+50, by vol.) Periodic acid+benzidine reagent (Smith, 1960) was used to detect the sugar alcohols.

Hexosamines. Hydrolysis of samples for the quantitative estimation of hexosamines was done in 4N-HCl at 100° for 4 hr. The hydrolysates were freed from interfering chromogens according to Boas (1958) and the total hexosamine content estimated by the method of Rondle & Morgan (1955). Colours were read at 530 m μ in a 1 cm. optical cell and compared with a glucosamine standard.

The individual amino sugars were separated on charcoal + celite columns (Perkins & Rogers, 1959) and muramic acid estimated by a micro modification of the Rondle & Morgan test. The reagents used in the micro modification were those described for the original test, but the final test volume was only 1.5 ml. instead of 10 ml. Extinctions were read at a wavelength of 505 m μ in a 2 cm. micro-cell after 24 hr and compared with a standard of authentic muramic acid (kindly supplied by Dr H. R. Perkins).

Agar-gel diffusion. Tests were made in a gel consisting of 1% (w/v) Ionagar no. 2 (Oxoid) in M/30 phosphate buffer (pH 7.2). Antigen preparations were suspended or dissolved in the same buffer. Antisera to staphylococci and L-forms were prepared in rabbits by a series of intravenous injections of whole organisms.

RESULTS

Hexosamines

Preliminary chromatographic evidence suggested that the amino sugar content of the L-forms was very much less than that of the parent staphylococci. In none of the four L-form strains was either muramic acid or glucosamine detected chromatographically, even when large (up to 10 mg.) samples of starting material were used. Large samples of L-form, which had not been extracted with cold 10% TCA prior to drying, yielded on hydrolysis and chromatography a minute quantity of a substance corresponding in R_f values to glucosamine. On acid-washing of the harvested L-form, however, this chromatographic spot was not detectable.

Separation of the total hexosamines from hydrolysates and quantitative estimation confirmed the results obtained by chromatography. Whereas the total hexosamine separated from hydrolysates of bacterial cells accounted for 2.79–3.8% of the dry weight of the organism, in hydrolysates of L-forms the amino sugars were not present in sufficient quantity to allow estimation. Consequently, the lower level of sensitivity of the test was taken as the maximum possible amino sugar content (Table 1).

Estimation of muramic acid content

The results above give total hexosamine content, but for the purposes of this investigation, estimation of the muramic acid content was of prime importance. Hydrolysates of both cocci and L-forms were treated on charcoal + Celite columns and the eluates that could be expected to contain muramic acid were collected. Estimation of the muramic acid contents of the eluates from L-forms gave very low values in the conventional Rondle & Morgan test, with glucosamine as a standard. Greater sensitivity was gained, however, by using the micro modification of the Rondle & Morgan test and by using authentic muramic acid as standard. In an effort to concentrate muramic acid further, the fractions of whole organisms,

Table 1. *Total hexosamine content of staphylococci and L-forms*

	Sample (mg.)	Hexosamine* content (μ g.)	Hexosamine as % dry weight
1444 cocci	10.18	284	2.79
L-form 1	12.20	<10	<0.082
2	26.50	<10	<0.038
PS6 cocci	10.62	309	2.91
L-form 1	7.10	<10	<0.141
2	31.20	<10	<0.032
PS42E cocci	9.65	279	2.89
L-form 1	6.70	<10	<0.149
2	28.80	<10	<0.035
Rogers cocci	2.50	95	3.80
L-form 1	6.70	<10	<0.149
2	12.50	<10	<0.080

* Material eluted by 2N-HCl from Dowex 50 H⁺, estimated in Rondle & Morgan (1955) test at 530 m μ and expressed as glucosamine base.

Table 2. *Muramic acid contents of isolated fractions from whole staphylococci and L-forms*

Organism	Residue from extraction	Equiv. dry wt. organisms (mg.)	Muramic acid recovered (μ g.)	Muramic acid in residue (%)	Muramic acid in whole organisms (%)
1444 cocci	S+H†	11.1	278*	13.9	2.50
	P+H	8.1	260	13.0	3.20
	Form	14.2	415	20.7	2.92
L-form	S+H	67.2	<4	<0.08	<0.006
	P+H	113.0	<4	<0.14	<0.004
	Form	56.1	<4	<0.12	<0.007
PS6 cocci	S+H	13.2	363	18.1	2.75
	P+H	17.8	593	24.7	3.33
	L-form				
	S+H	79.0	<4	<0.07	<0.005
	P+H	55.6	<4	<0.21	<0.007
PS42E cocci	S+H	12.2	327	16.3	2.68
	P+H	15.9	459	22.4	2.89
	L-form				
	P+H	47.9	<4	<0.24	<0.008
Rogers cocci	S+H	11.6	322	16.1	2.78
	P+H	12.5	406	20.3	3.25
	Form	10.5	317	15.9	3.02
L-form	S+H	53.4	<4	<0.25	<0.007
	P+H	55.5	<4	<0.04	<0.007

* Extraction methods used: Salton & Horne (1951), S+H; Park & Hancock (1960), P+H; Fuller (1938), Form.

† Estimated using the micro modification of Rondle & Morgan (1955) method; expressed in terms of a muramic acid standard.

whether cocci or L-forms, likely to contain the amino sugar were isolated. Hydrolysis of these fractions and estimation of their muramic acid contents gave the results shown in Table 2.

Since the results shown in Table 2 were obtained with preparations of L-forms which had been extracted with 10 % (w/v) TCA, there remained the possibility that

the L-forms might have contained small amounts of muramic acid in an acid-soluble form. Trichloroacetic acid was removed from the extracts with ether, and the aqueous solutions dried. Hydrolysis of the acid-soluble material and subsequent amino sugar estimation yielded no evidence of muramic acid.

Although the level of estimation of muramic acid was considerably decreased by using the micro modification, no detectable colour at 505 m μ was found in hydrolysates of fractions from L-forms. The maximum amount of muramic acid which could be present was taken as 4 μ g. By virtue of the characteristic spectrum of muramic acid in this test, lower values than 4 μ g. were detectable but could not be measured with certainty. Even so, the results obtained serve to show the absence of muramic acid and so of cell-wall mucopeptide from the L-forms of *Staphylococcus aureus*.

Cell-wall teichoic acid

The loss of mucopeptide during induction to the L-phase raises the question of the fate of the other major cell-wall polymer, the ribitol teichoic acid. If it were still produced, this teichoic acid could be found in two possible sites, still attached to the cell even in the absence of rigid mucopeptide, or it could be released into the medium as produced. Whole L-form preparations were hydrolysed to release any sugar alcohols that they might contain and the hydrolysates subjected to paper chromatography. At the same time, samples of whole cocci were similarly treated. Staining of the developed chromatograms with the periodic acid + benzidine reagent revealed the presence of spots corresponding to ribitol and anhydribose in hydrolysates of cocci. Small amounts of glycerol were also detected. Faint traces of glycerol but no spots corresponding to ribitol or anhydribose were evident in hydrolysates of L-forms.

Prolonged extraction of samples of L-forms with cold 10% (w/v) TCA, the normal procedure for extraction of teichoic acids, did not remove any material containing either ribitol or glycerol. The same treatment, when applied to the parent staphylococci, extracted an ethanol-insoluble substance, which on hydrolysis and chromatography yielded spots characteristic of both ribitol- and glycerol-teichoic acids.

Hydrolysates of freeze-dried supernatant medium from L-form cultures contained no detectable ribitol or anhydribose, but did contain traces of glycerol. In spite of this finding, prolonged extraction of the broth residue with cold TCA and attempts to precipitate glycerol teichoic acid proved fruitless.

Agar-gel diffusion

Further evidence for the lack of ribitol teichoic acid from the L-form strains was provided by gel diffusion experiments. Whereas suspensions of whole staphylococci or cell walls produced a characteristic 'polysaccharide A' precipitation line when diffused against antiserum to *Staphylococcus aureus*, suspensions of the L-forms did not. No 'polysaccharide A' was extracted from any of the L-form strains by the technique of Haukenes, Losnegard & Oeding (1961). The L-forms produced only what appeared to be a single line with the staphylococcal antiserum; and this line showed identity with one produced by intracellular contents isolated from the staphylococci.

Since the normal cell wall of the coccus had been so drastically altered during induction to the L-form, the normal means of serological identification were lost. However, trypsin extracts of membranes isolated from the four L-form strains, when diffused against L-form antiserum produced apparently single precipitation lines (Fig. 1). These lines showed fusion with each other, indicating the serological identity of the original membranes. Similar preparations of 42E staphylococcal cell membranes also produced a single precipitation line with L-form antiserum. Fusion of this line with that produced by a trypsin preparation of 42E L-form membranes (Fig. 2) established the similarity between the membrane structures of the staphylococcus and its L-form. This relationship held for all four strains of staphylococci and L-forms, and cross-reaction between the strains was complete.

Fig. 1

Fig. 2

Fig. 1. Agar-gel precipitin reactions of 1444 L-form antiserum (centre well) with trypsin extracts of membranes isolated from (A) 1444L, (C) 42EL, (D) 6L and (E) L Rogers. Well B contains a buffer suspension of whole 1444L-form and F a trypsin-buffer control.

Fig. 2. Precipitin reactions of 42E L-form antiserum (centre well) with (A) whole 42E L-form, (B) trypsin extract of 42E cell walls, (C) trypsin extract of 42E staphylococcal cell membranes and (D) trypsin extract of 42E L-form membranes.

DISCUSSION

The nature of the change produced in the cell wall of *Staphylococcus aureus* during induction to the L-form was indicated by Williams (1963), who described the osmotic sensitivity and insusceptibility to phage action in the staphylococcal L-form. These characters, together with the evidence of antibiotic sensitivities, presented in the same paper, pointed to an alteration in surface structure in the L-form, but did not of themselves provide proof of total lack of cell wall material. Sharp (1963) presented evidence for the absence of muramic acid from one strain of staphylococcal L-form, that of *S. aureus* H. The results of the chemical analyses for muramic acid content of the four staphylococcal L-forms agree with the findings reported by Sharp. However, a further indication of the extensive loss of cell-wall material from the L-forms is evident from the failure to find ribitol teichoic

acid in these organisms. Since the precise structural relationship between the cell-wall teichoic acid and the muramic-acid-containing mucopeptide is unknown, it is impossible to judge whether the non-production of ribitol teichoic acid might be caused by the absence of rigid mucopeptide or is a more direct consequence of antibiotic action.

In L-forms of group A streptococci, the M protein, which is normally found in the cell wall, is still produced but is liberated into the culture medium (Sharp, Hijmans & Dienes, 1957). The possibility was considered that the teichoic acid of the staphylococci might behave in the same way but no evidence for this was found.

With the absence of cell wall and the loss of characters such as cell-wall-associated antigens, Gram-staining properties and typical morphology, the identification of the L-forms as being derived from *Staphylococcus aureus* is difficult. Williams (1963) described the continued coagulase production by, and lysogenicity of, the L-forms. The present finding that the surface membrane of the L-form bears a close serological relationship to the cell membrane of the staphylococcus provides additional evidence on the origin of the L-form strains.

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