The Cellular Location of Antigens in Streptococci of Groups D, N and Q

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SUMMARY

The absence of Group D antigen from the cell walls and protoplasts of Group D streptococci leads to the conclusion that this antigen is located between the protoplast membrane and the cell wall. The Group N antigen was likewise not present in cell walls of Group N streptococci and probably has a similar location to the Group D antigen. The Group Q strains examined possessed the Group D antigen in the cell contents although these strains did not physiologically correspond to any established Group D species. The antigen upon which Group Q is based is a cell-wall antigen.

INTRODUCTION

Following the work of Elliott (1959) and Jones & Shattock (1960), who reported the unexpected absence of Group D antigen from the cell walls of Group D streptococci, the location of this antigen has been further investigated by examining bacterial bodies lacking cell walls. The presence of the antigen in these bodies would indicate a cytoplasmic location. The Group D antigen is now known to be a glycerol teichoic acid (Wicken, Elliott & Baddiley, 1963) and the cellular location is thus of special interest in relation to the function of these compounds.

Since bacterial protoplasts and L-forms lack cell walls they are suitable material for examining this problem. Hijmans (1962) examined L-forms of Group D streptococci and found no group or type (cell-wall) antigens or precursors in the L-forms or in the culture media. The L-forms used by Dr W. Hijmans (Leiden) were kindly supplied by him and used for some of the work reported here.

Lysozyme is commonly used for the production of bacterial protoplasts. However, *Streptococcus faecalis* (Group D) was originally reported to be resistant to the action of this cell wall-degrading enzyme (Salton, 1953). In our experience many Group D streptococci are sensitive to lysozyme; this has also been reported by Abrams (1959), Salton & Pavlik (1960), Bleiweis & Zimmerman (1961), Chesbro (1961), and Bibb & Straughn (1962). Because of some difficulties in preparing true lysozyme a phage-associated cell-protoplasts by using wall lysin (Bleiweis & Zimmerman, 1961) was also used in the present work.

Because of the physiological similarity of Group N and Group D streptococci it was decided to investigate the location of the Group N antigen by the technique used with the Group D antigen. Slade (1961) reported that with one Group N strain the group antigen was detected in the cell wall and in the residue after removal of

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cell walls from suspensions of disintegrated cocci. In the present work 17 strains of Group N streptococci, including *Streptococcus lactis* and *S. cremoris*, were examined. Group Q (established by Guthof, 1955) constitutes a group of streptococci which have many physiological resemblances to some species of Group D streptococci, and it was therefore of interest to investigate the cellular location of antigens in this group. Slade (1961) reported findings with two Group Q strains similar to his findings with the Group N strain.

METHODS

Organisms. Streptococci of Groups D, N and Q were obtained from the collection maintained in this laboratory. *Streptococcus lactis* strains ML 3 and c 10 and their phages were obtained from Mr B. Reiter (National Institute for Research in Dairying). The Group D phage was isolated from Reading sewage by the method of Adams (1950).

Physiological tests

The nutrient medium for growing organisms for physiological and serological tests was glucose Lemco broth (% w/v: Evans peptone, 1; Lab. Lemco, 1; NaCl, 0.5; glucose, 0.5). The temperature of incubation was 37° (30° for Group N streptococci). For physiological tests the inoculum was one loopful (4.0 mm. diam.) of 18-hr broth culture. L-forms of Group D streptococci were grown in the liquid medium described by Hijmans (1962).

Sugar reactions. Tubes of peptone water (1 %; 5 ml.) containing 0.5–1.0 % (w/v) test sugar, with bromocresol purple as indicator, were inoculated and examined daily for acid production.

Tolerance of potassium tellurite. Streak plates of glucose nutrient agar containing potassium tellurite (0.04 %, w/v; Skadhauge, 1950) were examined after incubation for 24 hr. Tolerant streptococci (*Streptococcus faecalis*) grew profusely as black colonies; no growth or a dusty grey growth of small colonies was recorded as negative.

Reduction of tetrazolium. The liquid medium of Barnes (1956) was used and examined for a red precipitate after incubation for 18 hr.

Methylene blue. Milk containing methylene blue (0.1 %, w/v) was inoculated with 0.2 ml. of 18-hr glucose Lemco broth culture and examined during 10 days for growth and dye reduction.

Growth at 50°. One loopful of an 18-hr broth culture was inoculated into 5 ml. glucose Lemco broth which was incubated in a water bath at 50°. The culture was examined for growth daily for 8 days.

Serological methods

Preparation of group-specific antisera in rabbits. The technique of Jones & Shattock (1960) was used for preparing Groups D and Q antisera. Group N antisera were prepared by using acetone-extracted ball-milled cocci as antigens (Shattock & Mattick, 1943).

Antigen extraction. The HCl-extraction procedure of Lancefield (1933) was most frequently used.

Precipitin-ring and absorption tests were done as described by Shattock (1949).

Antigen assay. Serial dilutions of antigen extracts in physiological saline were placed in the peripheral wells of slide gel patterns (Mansi, 1958), with antiserum in the central well. The greatest dilution of antigen which showed reaction with antiserum after diffusion for 3 hr was taken as endpoint; the antigen titre was the reciprocal of the endpoint dilution. In some tests the visibility of the precipitation bands was enhanced by immersing the slides in 0.0125 % (w/v) cadmium chloride solution (Crowle, 1958).

Analytical methods

Cell-wall isolation. Cell walls and 'cell contents' were isolated by the method of Cummins & Harris (1956), except that pepsin digestion was omitted.

Rhamnose determination. Rhamnose was assayed by the sulphuric acid + cysteine colour reaction of Dische & Shettles (1948).

Preparation of osmotically fragile bodies

Preparation of phage-associated enzymes. The method of Bleiweis & Zimmerman (1961) was followed for the isolation of phage-associated enzymes from *Streptococcus faecalis* var. *zymogenes* strain H 69 D 5 and *S. lactis* strains ML 3 and C 10.

Production of osmotic fragility. The organisms for spheroplast or protoplast production were grown in 5 ml. glucose Lemco broth for 18 hr; 1 ml. of this culture was then inoculated into 10 ml. glucose Lemco broth, this incubated for 5 hr and the whole added to 100 ml. warm glucose Lemco broth and incubated for a further 2-4 hr. The harvested organisms were washed twice in 0.85 % NaCl solution and suspended in the appropriate buffered solutions. The organisms from a 110 ml. glucose Lemco broth culture in the logarithmic growth phase were suspended in 80 ml. of 1.1 or 2.0 M-sucrose buffered at pH 6.2 with 0.15 M- K_2HPO_4 . Lysozyme (Armour Laboratories) was added to a concentration of 720 μ g./ml. In each experiment there were control suspensions without sucrose. Turbidity changes were followed by nephelometer readings. For experiments with phage-associated enzyme the same procedure was followed except that enzyme solution (4 %, v/v) was used in place of lysozyme.

RESULTS

Spheroplasts of Group D streptococci

Abrams (1959) reported the preparation of protoplasts from *Streptococcus faecalis* ATCC 9790 by using egg-white lysozyme to remove cell walls and 0.4 M-sucrose to stabilize the osmotically fragile bodies. Two cultures of this organism were obtained (one from Dr A. Abrams, University of Colorado; one from the National Collection of Industrial Bacteria, Aberdeen) and examined physiologically and serologically. The two cultures received, which were both labelled ATCC 9790, were not *S. faecalis* as defined, for example, by Shattock (1962); they resembled *S. faecium* or *S. durans* except that they did not grow in milk + 0.1 % methylene blue. HCl-extracts of these organisms reacted very weakly with Group D antiserum produced with *S. faecalis* strain s 161. This finding correlates with the different structure of the glycerol teichoic acid in this strain as compared to *S. faecalis* strain 39 (Wicken & Baddiley, 1963). It was concluded that the organism received as ATCC 9790 was an unsuitable strain for investigating the location of the Group D antigen.

When other Group D streptococci (in the logarithmic growth phase) were treated with lysozyme, osmotically fragile bodies were produced, but these did not have the appearance of true protoplasts when examined by phase-contrast microscopy. The cocci remained associated in pairs and short chains, indicating the presence of residual cell-wall material. These bodies were concluded to be spheroplasts, that is, osmotically fragile cocci with partially degraded cell walls. Rhamnose assays were impracticable because of the high sucrose concentration.

Table 1. *Group antigen loss from Group D streptococci suspended in saline and in concentrated sucrose, and during formation of spheroplasts by lysozyme*

	Whole organisms in 0.15 M-NaCl	Whole organisms in 1.1 M-sucrose, pH 6.2	Whole organisms producing spheroplasts in 1.1 M-sucrose pH 6.2 + lysozyme
	% diminution of antigen titre* in 2 hr at 37°		
<i>S. faecalis</i> var. <i>liquefaciens</i> Elv. 2025 Type 1	nt	20	55
<i>S. faecalis</i> var. <i>zymogenes</i> N 37 Type 1	0	15	50
<i>S. faecalis</i> var. <i>zymogenes</i> D 76 Type 1	15	35	60
<i>S. faecalis</i> 775 Type 6	25	65	75

nt, not tested; *estimated by gel-diffusion assay against a Group D antiserum prepared from *S. faecalis* s 161.

The Group D antigen in spheroplasts and in supernatant fluids was assayed during lysozyme digestion of *Streptococcus faecalis* var. *liquefaciens* strain Elv. 2025 suspended in 1.1 M-sucrose. Samples were removed from the digestion mixture at 30-min. intervals, centrifuged and the particulate fraction and the supernatant fluid fraction extracted in 0.05 M-HCl at 100° for 10 min. The two extracts were used for antigen assay. The results (Fig. 1) showed that about half the total extractable Group D antigen was released into solution when spheroplasts were produced from whole cocci.

Table 1 shows the results of similar experiments with three other Group D strains. *Streptococcus faecalis* strain 775 lost antigen into solution more readily than did the other strains even in the absence of lysozyme; this was characteristic only of saline-washed cocci.

Streptococcus faecium was more sensitive than *S. faecalis* to lysozyme but was not so suitable for our experiments because of its small content of Group D antigen. Antigen was released when spheroplasts were prepared, but quantitative experiments were not done. A cytoplasmic membrane fraction of *S. faecium* strain HGH 511 prepared by osmotic lysis of spheroplasts did not contain Group D antigen.

Protoplasts of Group D streptococci

A phage-associated enzyme was isolated by the technique of Bleiweis & Zimmerman (1961) from a *Streptococcus faecalis* var. *zymogenes* strain H 69 D 5 phage + host system. *S. faecalis* and varieties were very sensitive to this phage-associated enzyme, lysis occurring within a few minutes; but *S. faecium*, *S. durans*, *S. bovis* and *S. equinus* were not affected. The addition of cysteine hydrochloride was not

Fig. 1

Fig. 2

Fig. 1. Effect of lysozyme (720 µg./ml.) on the distribution of Group D antigen in *Streptococcus faecalis* var. *liquefaciens* Elv. 2025 suspended in 1.1 M-sucrose.

Fig. 2. Effect of phage-associated lysin (4%, v/v) on the distribution of Group D antigen in *Streptococcus faecalis* var. *liquefaciens* Elv. 2025 suspended in 1.1 M-sucrose.

necessary for the activity of the enzyme preparation. The lysates of bacteria produced by the phage enzyme contained no rhamnose (a cell-wall carbohydrate) sedimentable at 38,000 g; it was therefore possible that true protoplasts might be prepared by this method.

When suspended in 1.1 M-sucrose (pH 6.2) *Streptococcus faecalis* and varieties were not completely lysed by the phage enzyme. Phase-contrast microscopy revealed isolated spherical bodies which were concluded to be protoplasts; resuspension of these bodies in distilled water resulted in immediate lysis.

The distribution of Group D antigen between the particulate fraction (protoplasts)

and the supernatant fluid was followed during the treatment of *Streptococcus faecalis* var. *liquefaciens* strain Elv. 2025 with phage enzyme. Samples (5 ml.) were removed at intervals from a reaction mixture of substrate organisms suspended in 1.1 M-sucrose with 4 % (v/v) phage-enzyme solution. The samples were centrifuged at 38,000 g for 20 min. and the deposit extracted in 1 ml. 0.05 M-HCl at 100° for 10 min. The supernatant fluids were acidified with M-HCl to a final concentration of 0.05 M-HCl and heated at 100° for 10 min. The neutralized extracts were assayed by gel diffusion against a Group D antiserum. The results (Fig. 2) showed that, when cell wall was removed, about 85 % of the group antigen went into solution where it could be detected. Similar experiments with two other Group D organisms of the same serological type (type 1) gave comparable results; during protoplast formation from *S. faecalis* var. *zymogenes* strains N 37 and D 76, 90 and 80 %, respectively, of the Group D antigen went into solution. With *S. faecalis* 775 (type 6) 90 % of the group antigen similarly went into solution when protoplasts were prepared by using phage enzyme.

L-forms of Group D streptococci

Hijmans (1962) found that L-forms of Group D streptococci produced no Group D antigen when grown in liquid medium containing penicillin. This observation was confirmed in the present work. It was also found that two penicillin-resistant strains of normal streptococci (*Streptococcus faecium*) did not produce the Group D antigen when grown in the L-form medium, but removal of the penicillin stimulated production of the antigen. It was therefore possible that it was the penicillin in the medium which was responsible for the absence of Group D antigen from the L-forms. The L-forms (nos. 27, 30) were stable in the absence of penicillin; no Gram-positive material was detected in the L-forms and there was no synthesis of rhamnose, a carbohydrate characteristic of streptococcal cell walls. When grown in a medium without penicillin and with 0.5 % glucose (a concentration favourable to Group D antigen production; Medrek & Barnes, 1962) instead of the usual 0.1 % glucose, one of the L-forms (no. 27) produced Group D antigen. After about six subcultures in penicillin-free medium L-form no. 27 lost ability to synthesize Group D antigen although it still produced an antigen which reacted with Group D antiserum but did not give a pattern of identity with Group D antigen in gel-diffusion tests. When the Group D antigen was produced quantitative tests showed that 96 to 98 % of the D antigen was free in the culture medium.

Cell-wall isolation from Group N streptococci

Cell walls were isolated from 17 strains of *Streptococcus lactis* and *S. cremoris*. HCl-extracts of the cell-wall fractions in no case reacted with Group N antiserum in gel-diffusion tests. The clear supernatant fluids obtained on centrifuging disintegrated coccal suspensions were rich in Group N antigen (Table 2).

Spheroplasts of Group N streptococci

All 20 strains of *Streptococcus lactis* and *S. cremoris* tested were lysed by egg-white lysozyme and by phage-associated enzyme isolated from *S. lactis* strains ML 3 and c 10 host + phage systems; complete release of rhamnose into solution was not achieved with either enzyme preparation. The stabilized osmotically fragile bodies

were therefore considered to be spheroplasts. Phase-contrast microscopy showed that the osmotically fragile bodies were usually still in pairs or short chains. Spheroplasts of *S. lactis* strain Rice prepared with lysozyme contained the same amount of Group N antigen as did corresponding whole cocci. Table 3 shows the

Table 2. Serological reactions of HCl extracts of whole cells, cell walls and 'cell contents' of Group N streptococci

Group N antiserum: prepared by using *S. lactis* OJ as immunizing strain.

	Whole organisms		Cell walls		Cell contents	
	Ring-test	Gel-diffusion test	Ring-test	Gel-diffusion test	Ring-test	Gel-diffusion test
<i>S. lactis</i> strains						
OJ	+	+	—	—	+	+
M1	+	+	—	—	+	+
H201	+	+	±	—	+	+
478 dem	+	+	nt	—	+	+
Rice	+	+	±	—	+	+
H240	+	+	—	—	+	+
H211	+	+	—	—	+	+
M8	+	+	—	—	+	+
85 Edin	+	+	—	—	+	+
92/4	+	+	—	—	+	+
UD Edin	+	+	—	—	+	+
<i>S. cremoris</i> strains						
198/1	+	+	—	—	+	±
95/2	+	+	—	—	+	+
98/54	+	+	—	—	+	+
236/M1	+	+	±	—	+	+
133/5	+	+	—	—	+	±
RW	+	+	—	—	+	+

nt, not tested; + (ring test), heavy ring within 5 min.; ± (ring test), slow but definite ring; ± (ring test), trace of reaction in 20 min.; + (gel diffusion), strong reaction, pattern of identity with other Group N HCl-extracts; ± (gel diffusion), weaker but definite reaction.

Table 3. Group N antigen content of normal organisms and spheroplasts of *Streptococcus lactis* and *S. cremoris*

	Whole organisms in sucrose*	Spheroplasts (c10 enzyme)	Spheroplasts (ML3 enzyme)
	Antigen titres		
<i>S. lactis</i> Rice	12	12	8
<i>S. lactis</i> c10	10	8	4
<i>S. cremoris</i> KH	3	1	3
<i>S. cremoris</i> E8	2	1	1

* 1.1 M-sucrose for *S. lactis*; 2 M for *S. cremoris*.

antigen titres of four Group N strains before and after spheroplast production; between 0 and 60 % of the group N antigen went into solution when the spheroplasts were formed.

Physiology of Group Q strains

The five Group Q strains examined did not correspond physiologically with any established Group D species although they resembled them in several ways (growth at 10°, 45°; in 6·5% NaCl; at pH 9·6; fermentation of several carbohydrates). As is seen in Table 4 the Group Q strains had characteristics intermediate between those of *Streptococcus faecalis* and *S. faecium*. Group Q streptococci typically ferment sorbose, but this ability is shared by some Group D and Group N strains: *S. faecium* strain OJ, *S. bovis* strains Lottie 15 and H 6, *S. lactis* strain 84 Edin.

Table 4. *Comparison of physiological characters of Streptococcus faecalis* 775, *S. faecium* HGH 511 and Group Q strains

	<i>S. faecalis</i>	Group Q	<i>S. faecium</i>
Reduction of tetrazolium	+	—	—
Tellurite (0·04 %) tolerant	+	—	—
Hydrolysis of Na hippurate	+	—	—
Acid from melezitose	+	+	—
sorbitol	+	+	—
rhamnose	+	+	—
glycerol (anaerobic)	+	+	—
sorbose	—	+	—
arabinose	—	+	+
Methylene blue (0·1 %) tolerant	+	—	+
Growth at 50°	—	—	+

Reaction of Group Q strains with Group D antisera

HCl-extracts of the five Group Q strains examined all gave positive precipitating reactions with Group D antisera. The extracts gave patterns of identity with HCl-extracts of Group D strains in gel-diffusion tests against Group D antisera. Strain E 6556 gave an exceptionally strong reaction with Group D antisera; the speed and intensity of the reaction were comparable to the results with *Streptococcus faecalis*. The other strains reacted less strongly and it was usually necessary to incubate the cultures for 48 hr before extraction. The reaction was enhanced by concentrating the extracts by the method of Shattock (1949). The Group Q strains were able to absorb Group D antibodies although the minimal absorbing doses were one to six times greater than with the homologous organism.

Antisera prepared by the inoculation of whole-cell vaccines of Group Q streptococci gave precipitin reactions only with Group Q strains. However, when disintegrated-cell vaccines were used (as for Group D) the resulting antisera reacted with extracts of Group Q and Group D strains. Gel-diffusion analysis showed that the reaction was due to two independent antibodies. Both antibodies were completely absorbed by Group Q organisms, while only the antibody which reacted with Group D extracts was absorbed by Group D organisms. The Group Q antigen was apparently equivalent to the type antigens of Group D. Extracts of the Group Q strains did not react with any of the Group D serological type antisera of Sharpe & Shattock (1952). Strain E 6556, identical in physiology with the other Group Q strains, did not react with Group Q antisera. Two cultures of this strain from different sources gave similar results.

Location of antigens in Group Q streptococci

Suspensions of Group Q organisms were disintegrated and cell walls and cell contents separated by centrifugation at 800 *g* for 60 min. HCl-extracts of cell contents and of cell walls were examined for the presence of Group D and Q antigens; the results are given in Table 5. The Group Q antiserum used in this experiment was prepared by using as vaccine formolized whole organisms of strain E 6749; this antiserum gave no reaction with HCl-extracts of Group D organisms.

Table 5. *Precipitin-ring tests of HCl-extracts of cell walls and HCl-treated 'cell contents' of Group Q streptococci*

	HCl-treated cell contents 100°/10 min.		HCl-extract of cell walls	
	Antiserum		Antiserum	
	Gp D	Gp Q	Gp D	Gp Q
E 6749	+++	±	—	+++
Q 2	+++	±	—	+++
E 6844	+++	+	—	+++
E 1551	+++	±	—	+++
E 6556	+++	—	—	—

+++ , Heavy ring within 5 min. ; + , moderate ring within 20 min. ; ± , slight ring within 20 min. ; — , no reaction.

DISCUSSION

Shattock's (1949) demonstration that Group D streptococcal antiserum production was improved when vaccines of disintegrated organisms instead of whole organisms were used was evidence for the internal location of the Group D antigen. McCarty (1952*a, b*) showed that the Group A antigen was an integral part of the cell wall and other group antigens were expected to be analogous. However, Elliott (1959) and Jones & Shattock (1960) reported that Group D antigen was not present in the cell walls. The latter authors examined 21 strains of streptococci representing all the species of Group D then recognized (*Streptococcus faecalis* and varieties, *S. faecium*, *S. durans* and *S. bovis*): all these strains lacked Group D antigen in their cell walls.

Elliott (1959) and Jones & Shattock (1960) were of the opinion that the Group D antigen had a cytoplasmic location. Elliott (1960) considered the antigen to be superficially located because of its easy extraction with cold alkali. The present observation that most of the Group D antigen went into solution when protoplasts were prepared, or when L-forms synthesized the antigen, appears to exclude the possibility of a cytoplasmic location. Slade & Shockman (1963) found that isolated protoplast membranes of '*Streptococcus faecalis* ATCC 9790' contained Group D antigen but they did not state whether the antigen associated with the protoplast membrane constituted all the antigen in the cocci.

The observation that Group D antigen was released as the cell wall was removed is considered to be evidence that the D antigen is situated at the surface of the protoplast, beneath the cell wall; this view was expressed by Shattock & Smith (1963). Similar conclusions were drawn by Wicken *et al.* (1963), again with '*Streptococcus faecalis* ATCC 9790'.

From the present results with *Streptococcus lactis* and *S. cremoris* it seems that the Group N antigen has a similar location to the Group D antigen; but the evidence is not conclusive since true protoplasts were not prepared nor were L-forms available. It remains to be seen whether the Group N antigen, like the Group D antigen, is a glycerol teichoic acid.

The five Group Q strains examined formed an homogeneous physiological group which did not correspond to any known species. However the group resembled the 'enterococcus group' of Sherman (1937), with characteristics intermediate between *Streptococcus faecalis* and *S. faecium*. Group Q strains showed serological cross-reactions with Group D strains. The D antigen present in the Group Q strains was absent from the cell walls and possibly is located, as in Group D streptococci, between the cell membrane and the cell wall. The Group Q antigen was detected principally in the cell-wall fraction and therefore corresponds in location with the type antigens of Group D. The serological cross-reaction of Groups D and Q has been observed also by Dr R. Wahl (personal communication).

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