A MUCOID FORM OF MICROCOCCUS PYOGENES VAR AUREUS WHICH SHOWS CAPSULAR SWELLING WITH SPECIFIC IMMUNE SERUM

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Received for publication October 12, 1953

There is but scanty reference in the literature to mucoid or encapsulated strains of *Micrococcus pyogenes* var *aureus* (*Staphylococcus aureus*). Porter and Pelczar (1941) in studying nutrition of staphylococci noted that two of their strains produced "extraordinarily mucoid" colonies when cultivated on blood agar. Bigger *et al.* (1927) reported "very viscid" colonies as variants of staphylococci. Gilbert (1931) and Oesterle (1936) described mucoid and encapsulated strains. Lyons (1937) also encountered encapsulated forms; in his experience they occurred in nonpathogenic as well as in pathogenic strains. He endeavored to produce the "quellung" reaction without success. This, as far as we can determine, is the only reference to capsular swelling of staphylococci in the literature. During the past year we have studied mucoid and viscid variants of a strain of *Staphylococcus aureus* which are encapsulated and show capsular swelling on treatment with specific immune serum. It is the purpose of this communication to describe these phenomena.

MATERIALS AND METHODS

The parent strain, designated "RL", was obtained from a throat culture taken on a patient under treatment for staphylococcal pneumonia. Fluid cultures were carried in nutrient broth, either plain or enriched with 2 per cent rabbit's blood, and colonial morphology was studied on 5 per cent rabbit's blood agar plates.

The mucoid variant appeared when the parent strain was passed through embryonated eggs with influenza virus. This virus was the "Bishop" strain, isolated in 1951, and was not mouse-adapted. It was classified as an A-prime strain. Eleven day chick embryos were inoculated by the allantoic route, 0.25 ml of a mixture of virus and staphyloccocal culture, each diluted to 10^{-3}, being injected.

Coagulase tests were performed with 0.5 ml of human plasma diluted 1:5 in saline and incubated with 0.1 ml of a 24 hour broth-culture of the organisms. After three hours of incubation in a 37 C water bath, they were held overnight at room temperature. Sensitivity to antibiotics was determined by the paper disc method.

Virulence was tested by the intraperitoneal inoculation of mice with varying dilutions of a 24 hour broth-culture of the organism suspended in an equal volume of 5 per cent gastric mucin in saline (Anderson and Oag, 1939). Pathogenicity was estimated also by the intramuscular inoculation of mice with 4 hour cultures (Selbie and Simon, 1952).

Capsular swelling was demonstrated in wet preparations. A small loopful of a 3 to 6 hour broth-culture, or of a saline emulsion from a plate, was mixed with a small loopful of methylene blue, and a large loopful of undiluted immune serum was added. The preparation was preserved with a coverslip and examined at various intervals thereafter. Immune serum was prepared by injecting rabbits intravenously with a heavy suspension of organisms from a 24-hour plate in saline killed either by heating to 60 C for one hour or exposure to 0.5 per cent formalin. Injections of 1.0 ml were given daily for 3 days followed by a 4 day rest period for 3 to 4 weeks.

RESULTS

On prolonged cultivation the colonial morphology of the parent strain "RL" has remained unchanged. Its appearance on blood agar is typical of *S. aureus*. It shows an abundant, smooth growth, can be emulsified easily in saline, and produces an orange pigment. Occasional albus mutants are observed. It is mannitol and coagulase positive. When exposed to antibiotics it was found to be sensitive to streptomycin (10 μg), slightly sensitive to penicillin (10 units) and bacitracin (20 units), and completely resistant to aureomycin (20 μg) and terramycin (100 μg).

After two plate and eight broth transfers, strain RL was inoculated into chick embryos by the allantoic route with influenza virus as previously described. Twenty-four hours later one of the eggs was opened, and the allantoic fluid was harvested and plated. *Staphylococcus aureus* grew abundantly on the plate, and wedges were seen in the growth that had a granular appearance. On subculture all the colonies were strikingly different from the parent strain. Individual colonies were surrounded by a wide mucoid zone, and this was so marked in confluent areas that the general appearance resembled that of *Klebsiella pneumoniae* (except, of course, for the color). This mucoid growth looked watery to the eye but actually was very tenacious, difficult to scrape off, hard to emulsify, and gave a very granular suspension. On repeated subculture the strain retained its mucoid character for a considerable period, but after 18 to 20 transfers it began to lose it. Under these circumstances the fully mucoid character can be restored completely by a single passage through the chick embryo without added influenza virus. Mucoid cultures in the frozen state preserve their character indefinitely. The strain grows equally well anaerobically.

The mucoid strain was found to be mannitol positive and coagulase positive although the clot is not so firm as that produced by strain RL. Its antibiotic sensitivities are identical to those of strain RL. Moreover, phage typing was performed on both the RL strain and the mucoid strain by Dr. Blair, and an identical pattern was found for each, i.e., both were lysed by phages 44a and 52a. For these reasons we concluded that the mucoid strain was a variant of strain RL and have designated it RL-mucoid.

The virulence of the RL-mucoid variant was also compared with that of the parent strain. Neither will kill mice after an intraperitoneal inoculation of 0.25 ml of a 24 hour broth culture unless mucin is added. With mucin death occurs, and the organism can be recovered from the heart's blood. Titrations with this technique reveal no difference in virulence between the two. Nor could any differences be found by the technique of intramuscular inoculation of mice. It is of interest that both the RL-mucoid and RL strain retain their original characteristics when cultured from dead mice.

The gross appearance of the RL-mucoid variant immediately suggested the presence of a large amount of capsular substance. As capsules could not be demonstrated satisfactorily by the usual, dry, staining methods, we prepared rabbit antiserum in order to see whether the quellung phenomenon could be produced. Using RL-mucoid vaccine made according to the method described above, a serum was prepared which gave unequivocal capsular swelling in the wet preparation stained with methylene blue (see figure 1). This reaction does not take place quite as rapidly as in the case of the pneumococcus. It comes up regularly, however, in 1 to 3 hours and is best demonstrated in young cultures. With 24 hour cultures capsular swelling appears only after about 18 hours and is less striking. The reaction is equally good when organisms are grown in the presence of blood or serum, but is destroyed by heat or formalin. Perhaps because of the age of the growth, it has not been demonstrated with organisms taken directly from the peritoneal exudate of mice or the allantoic fluid of infected embryos. The RL-mucoid variant antiserum is active whether produced by heat killed or formalin treated vaccine. The quellung reaction has never been produced with normal rabbit serum nor by exposure to stock, staphylococcus antiserum, types A and B.

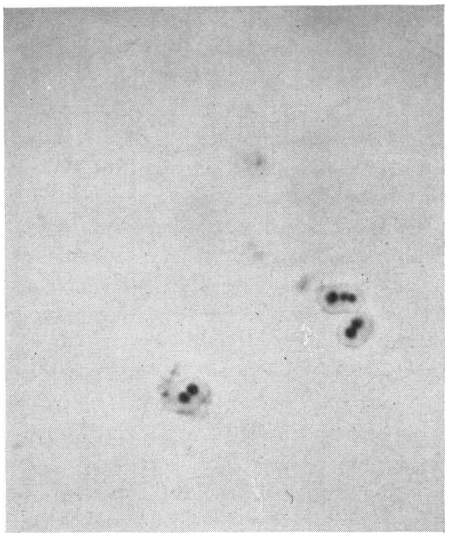

Figure 1. Capsular swelling of staphylococci (× 1,000). Three hour broth culture of RL-mucoid organisms in RL-mucoid antiserum. Photomicrograph taken after a three hour exposure.

We have endeavored to produce another RL-mucoid variant on several occasions by the method described above, but thus far without success. While another mucoid variant has not appeared, we have observed regularly that when strain RL is cultivated in allantoic fluid, with or without influenza virus, a change in colonial morphology takes place. The colonies become "viscid". They are not watery looking but are tenacious as in the case of RL-mucoid variant, and they, too, emulsify poorly. We have designated this variant as RL-viscid.

Antisera have been prepared against all three strains and cross tested. When RL-viscid organisms are exposed to RL-mucoid antiserum, capsular swelling takes place, but it is somewhat slower and less striking. Anti-RL-viscid serum produces capsular swelling of mucoid organisms, but on the whole our strongest sera have been obtained from the RL-mucoid variant. Lastly, anti-RL serum, prepared after a more prolonged course of immunization, will produce some capsular swelling of mucoid organisms. This serum is the weakest of the three varieties. When staphylococci of the parent RL strain are exposed to strong anti-RL-mucoid serum, no immediate capsular swelling is seen. Overnight, however, an occasional organism shows some capsular swelling. From a quantitative standpoint the reaction is unimpressive. These relationships are indicated schematically in table 1.

Another interesting feature of the RL-mucoid variant is its capacity to agglutinate erythrocytes, a property not shared by the viscid or parent strains. Agglutination of fowl red cells with blood-broth cultures occurs regularly. Human and chick embryo cells are agglutinated less regularly, and the reaction is more likely to occur with a strain recently passed through eggs. Unlike the agglutination of erythrocytes by influenza virus, this reaction is prevented by formalin and shows no specific inhibition by anti-influenza serum.

TABLE 1

Schematic representation of the capsular swelling phenomenon with three antisera against the parent strain of staphylococci and its mucoid and viscid variants

SERUM	ORGANISMS		
	RL-mucoid	RL-viscid	RL
Anti-RL-mucoid	++++	+++	±
Anti-RL-viscid	+++	++	0
Anti-RL	+	0	0

The symbols are a rough indication not only of the apparent size of the capsules and the number of organisms involved but of the speed with which the reaction occurs

DISCUSSION

We have described mucoid and viscid variants of *Staphylococcus aureus* and have included what we believe is the first recorded example of the capsular swelling phenomenon in connection with this organism. A review of the literature indicates that in all probability strains similar to our RL-mucoid variant were noted by Gilbert (1931) and Oesterle (1936). The source of Gilbert's strain was peritoneal and pericardial fluids in a patient dying of gonococcal endocarditis. The origin of Oesterle's strain is more obscure; it was said to have come from pus, the source of which was not known. We cannot explain the significance of the fact that our RL-mucoid variant was produced by passage in embryonated eggs with influenza virus, nor why we have been unable to repeat this transformation. The fact that only a few other mucoid variants have been described suggests that the transformation may be a fortuitous one. Our RL-viscid variant resembles the description of viscid variants of staphylococci by Bigger *et al.* (1927). We were unable to demonstrate any difference in animal virulence between the RL-strain and its mucoid variant although such was not the experience of Gilbert. Her mucoid strain was virulent for guinea pigs, and its nonmucoid variant was avirulent.

We believe that the evidence presented in this paper indicates that the difference between RL, RL-viscid, and RL-mucoid strains is related quantitatively to the amount of capsular substance produced. Capsular substance production of the parent strain RL is relatively small, that of RL-viscid moderate, and that of RL-mucoid marked. This is borne out by the difference in the degree of capsular swelling when strong antiserum is applied to the three strains. Moreover, the fact that RL-mucoid vaccine produces strong, swelling-producing serum, and RL-viscid a serum of moderate strength, whereas anti-RL serum is

the weakest of the three, suggests that the presence of abundant capsular substance in the antigen is necessary for the production of a potent antiserum. Whatever is the serum, the capsular swelling reaction is always most striking with the mucoid variant in the fully mucoid stage.

The serological specificity of this reaction remains to be determined since we have not had the opportunity to study the capsular substance from a variety of different strains of staphylococci to find out whether they are antigenically distinct. If this should be the case, it would create a new method for serological classification. Further work along this line is in progress. As it may be relatively easy to produce viscid forms by egg passage, it should be possible to collect an adequate supply of material in a form suitable for study by the quellung technique. Moreover, as antiserum against our parent strain RL produced by hyperimmunization gives some capsular swelling of the mucoid variant, this technique could be used also for serological comparison.

ACKNOWLEDGMENTS

We are indebted to Dr. Thomas Magill for classification of the Bishop strain of influenza virus. The phage typing of our parent and mucoid variant was kindly performed by Dr. John E. Blair. The stock antistaphylococcal sera were obtained through the kindness of Dr. D. Khorazo. The photomicrograph was prepared by Dr. S. Ellison.

SUMMARY

Mucoid and viscid variants of a strain of *Micrococcus pyogenes* var *aureus* (*Staphylococcus aureus*) are described. These are encapsulated.

Antiserum prepared against encapsulated strains produces the quellung phenomenon with encapsulated organisms.

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