Immunofluorescence Examination of Typical *Staphylococcus aureus* Variants and L-forms for Clumping Factor

By K. L. ELKINS, R. M. HYDE AND FLORENE C. KELLY

*Department of Microbiology, University of Oklahoma Medical Center,
Oklahoma City, Oklahoma 73104, U.S.A.*

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SUMMARY

Encapsulated coagulase-positive staphylococci which do not clump with fibrinogen possess the clumping factor antigen as well as other cell-wall antigens characteristic of *Staphylococcus aureus*. Variants which were negative for both coagulase and clumping factor not only lacked the clumping factor antigen but also other *S. aureus* surface antigens. L-forms did not remove clumping inhibiting antibody from *S. aureus* antisera. Antisera which were relatively high in clumping-inhibiting antibodies, but low in agglutinins, gave no fluorescence with coagulase-negative, clumping factor-negative variants or L-forms. The clumping factor, probably a cell-wall component of *S. aureus*, is absent in the penicillin-induced, stable L-form.

INTRODUCTION

The characteristic clumping of coagulase-positive staphylococci in the presence of fibrinogen is attributed to a factor (bound coagulase or clumping factor) which is presumed to be a surface component of the cocci. Duthie (1954) reported that an antigen of *Staphylococcus aureus*, which was released into the culture medium upon autolysis, induced production of antibodies which inhibited the clumping reaction. This antigen has not been detected in staphylococcal extracts which precipitate fibrinogen (Rotter & Kelly, 1966; Blackstock, Hyde & Kelly, 1968). Kato & Omori (1959) verified the observation of Duthie (1954) that trypsinized *S. aureus* cocci lose the ability to clump in fibrinogen solution, and they no longer absorb the clumping-inhibiting antibodies from antiserum. It was assumed that the release of clumping factor into the medium of ageing cultures, as well as its loss upon refrigeration and trypsinization, is probably due to a 'peeling off' of cell-surface substance. Gorrill, Klyhn & McNeil (1966) found that clumping factor was either destroyed or removed by sonic vibrations. A remnant of cell-wall material may be present on some or all of the so-called L-forms of *S. aureus* (Pratt, 1966; Hamburger & Carleton, 1966); clumping factor could be a component of this cell-wall remnant. Coagulase and clumping factor are closely associated in the bacterial form of typical *S. aureus* strains, and L-forms of these strains are known to produce coagulase (Mattman, Turnstall & Rossmoore, 1961).

In this immunofluorescence study typical coagulase-positive cocci, clumping factor-negative and L-forms of *Staphylococcus aureus* were tested with antisera of known agglutinating and clumping-inhibiting antibody content.

METHODS

Staphylococcal strains. Six typical *Staphylococcus aureus* strains were used: 2095, 3189 and 2253, strains of known serological type (Cohen & Smith, 1964) supplied by Dr J. O. Cohen; J17, serologically of Cowan's group I (from Dr K. Jensen, who used it in his antigen A studies: personal communication); 604, a wild strain isolated here; the SMITH strain (from Dr G. A. Hunt). Also used were: the coagulase-negative, clumping factor-negative variants of strains J17 and 604 designated J17M and 604M, respectively; strain sv, an encapsulated, mouse-virulent variant of the SMITH strain (Hunt & Moses, 1958); and K6, an encapsulated, mouse-virulent strain which was isolated here from the nasopharynx of a healthy carrier in 1956. Although coagulase-positive, neither strain sv nor strain K6 clump in fibrinogen solutions.

Induction and cultivation of L-forms. Five coagulase-positive, clumping factor-positive strains (2095, 2253, 3189, 604 and J17) were induced to L-form growth. The inducing medium was similar to that used by Marston (1961a) and Williams (1963). A concentrated inoculum was prepared by centrifuging a 5 to 8 hr Brain-Heart-Infusion (Difco) broth culture (37°) and resuspending the cocci in one-fifth the original volume of broth. One-half millilitre (0.5 ml.) was plated on Brain-Heart-Infusion (Difco) medium which contained 1 % (w/v) Special Agar-Noble (Difco), 5 % (w/v) sodium chloride, 10 % (v/v) horse serum and 1500 units penicillin G/ml. L-colonies, which developed after (37°) incubation, were subcultured first on the inducing agar medium and thereafter in a similar liquid medium. Once stable L-form growth was obtained, subsequent broth cultures were shaken at low speed on a Burrell wrist action shaker during a 48 hr incubation period at 37°. L-forms were also grown in soft agar medium prepared by adding 0.15 % (w/v) Special Agar-Noble (Difco) to the liquid medium. Modifications of the soft agar medium included addition of 4 % (w/v) human fibrinogen (Merck, Sharp & Dohme, West Point, Pa.) or bovine fibrinogen (Armour Fraction I, Armour Pharmaceutical Co., Kankakee, Ill.) for testing clumping factor activity, and incorporation of 1 % (v/v) rabbit plasma in the fibrinogen-soft agar for demonstrating free coagulase (Alami & Kelly, 1959).

Preparation and use of antisera. Immunization of rabbits with typical *Staphylococcus aureus* strains began with two 0.5 ml. intravenous injections of formalinized (2×10^9 cocci/ml.) vaccine in the first week, and was continued, for a total of 8 weeks, with similar, biweekly injections of cocci which had been heated at 65° for 2 hr. Less than 1 % of the heated cocci were viable.

Samples of each antiserum were absorbed with clumping factor-negative *Staphylococcus aureus* variants and with L-forms. L-forms separated from a 300 ml. broth culture were washed once in 3 % (w/v) NaCl solution and antiserum was added to the sedimented growth. Absorption, with washed typical cocci or L-forms, was allowed to proceed at 37° for 2 hr and at 4° for an additional 24 hr. Antisera against three strains were absorbed with lyophilized supernatant fluid of the broth culture in which the L-form had grown, as well as with lyophilized, uninoculated, L-form broth medium. Antisera were held at -21° until used in agglutination, clumping inhibition and indirect fluorescent-antibody tests. In immunodiffusion experiments antiserum was substituted for horse serum in L-form agar medium.

Indirect fluorescent-antibody tests. Fluorescein isothiocyanate-labelled sheep anti-rabbit gamma globulin (Colorado Serum Company, Denver, Colorado) was applied

to cocci and L-forms which had been reacted with pre-immune, unabsorbed immune or absorbed immune rabbit serum. In a control to rule out nonspecific fluorescence, incubation of organisms with antiserum was omitted from the procedure. Slides were made of staphylococci which had been separated from an 18 hr Brain-Heart-Infusion (Difco) culture (37°), washed once and resuspended in saline. Initially, the method used by Karakawa, Rotta & Krause (1965) in immunofluorescence studies of streptococcal L-forms was applied. However, the most satisfactory preparations of staphylococcal L-forms proved to be smears of growth from broth cultures or of colonies from soft (0.15 %) agar cultures. Slides were examined under a Reichert u.v. microscope using a BG-12 exciter filter and an OG-1 barrier filter.

RESULTS

Examination of L-forms for coagulase and clumping factor. The L-form was considered stable when no bacterial colonies developed on penicillin-free medium. The sedimented growth of broth cultures inoculated with L-colonies contained numerous large bodies similar to those described by Marston (1961*b*), Weibull (1963) and Williams (1963). Occasionally, in broth cultures, the L-form gave rise to small discrete colonies which, on transfer to fresh broth, produced the usual diffuse growth. Typical L-colonies developed from either the diffuse growth or the discrete colonies.

Coagulase-production by L-forms was demonstrated by the plasma-fibrinogen-soft agar method as well as by use of broth cultures in the regular plasma clotting test. Clumping factor activity could not be detected in fibrinogen-soft agar because compact colonies sometimes developed in the fibrinogen-free soft agar base. The viscous nature of the L-form growth, on agar or in broth, made it unsuitable for use in the slide clumping test usually applied to staphylococci.

Table 1. *Homologous agglutination titres before and after absorbing Staphylococcus aureus antisera with variants and L-forms of S. aureus*

<i>S. aureus</i> antiserum (immunizing strain)	Unabsorbed antiserum	Antiserum absorbed with				L-form of immunizing <i>S. aureus</i> strain
		Cocci (strain)				
		J17M*	604M*	sv†	K6†	
J17	1/2560	1/1280	1/2560	—‡	1/10	1/1280
604	1/2560	1/2560	1/1280	—	1/10	1/1280
3189	1/5120	1/2560	1/2560	1/10	1/20	1/2560
2253	1/1280	1/1280	1/1280	—	—	1/320
2095	1/2560	1/2560	1/2560	1/10	1/20	1/1280
S	1/1280	1/640	1/640	—	1/10	1/640

* J17M and 604M are coagulase-negative, clumping factor-negative variants of *S. aureus* strains J17 and 604, respectively.

† sv and K6 are encapsulated, coagulase-positive strains which do not clump in fibrinogen.

‡ No reaction observed in lowest serum (1/10) dilution tested.

Agglutination and clumping inhibition reactions. Prior to immunization, sera of rabbits were negative for clumping-inhibiting antibody and in no instance was the agglutination titre for strains used higher than 1/10. Titres of immune sera ranged

from 1/1280 to 1/5120 for agglutinating antibody and from 1/320 to 1/2560 for clumping-inhibiting antibody.

There was no significant change in either reaction as a result of absorption with coagulase-negative, clumping factor-negative (J17M or 604M) cocci, nor did L-form absorption have any appreciable effect, except in the case of strain 2253. In the three instances tested (strains 604, 3189 and 2095) absorption of antiserum with the concentrated supernatant fluid of L-form broth cultures caused no change in titres. The encapsulated, coagulase-positive, clumping factor-negative (sv or K6) cocci removed both agglutinating and clumping-inhibiting antibodies. However, as a result of absorption with these cocci there was a greater decrease in agglutination titres than in clumping inhibition titres.

Tables 1 and 2 show the agglutination and clumping inhibition titres, respectively, of antisera after repeated absorptions with variant staphylococci and with L-forms of the immunizing strain.

Table 2. *Homologous clumping inhibition titres before and after absorbing Staphylococcus aureus antisera with variants and L-forms of S. aureus*

<i>S. aureus</i> antiserum (immunizing strain)	Unabsorbed antiserum	Antiserum absorbed with				L-form of immunizing <i>S. aureus</i> strain
		Cocci (strain)				
		J 17 M*	604 M*	sv†	K 6†	
J 17	1/2560	1/1280	1/2560	1/160	1/320	1/1280
604	1/1280	1/1280	1/1280	1/20	1/40	1/640
3189	1/2560	1/2560	1/2560	1/160	1/640	1/1280
2253	1/320	1/320	1/320	1/20	1/40	1/160
2095	1/640	1/640	1/640	1/80	1/160	1/320
s	1/2560	1/1280	1/2560	1/320	1/640	1/1280

* J17M and 604M are coagulase-negative, clumping factor-negative variants of *S. aureus* strains J17 and 604M, respectively.

† sv and K6 are encapsulated, coagulase-positive strains which do not clump in fibrinogen.

Immunodiffusion reactions of L-forms. A zone of precipitate surrounded L-colonies on agar medium containing homologous *Staphylococcus aureus* antiserum. Absorption of the antiserum with cocci of the coagulase-negative (J17M or 604M) variants had no observable effect on this reaction. No precipitation developed when the antiserum was absorbed with typical *S. aureus* or with the encapsulated, coagulase-positive, clumping factor-negative (sv or K6) cocci.

Fluorescent-antibody reactions. Results with strain J17, as presented in Table 3, are typical of those obtained when other *Staphylococcus aureus* strains were tested with homologous antisera.

Pre-immune serum caused slight fluorescence of coagulase-positive strains, regardless of their clumping activity, but no fluorescence of the coagulase-negative variants or L-forms was observed. Fluorescence of coagulase-positive staphylococci was eliminated by absorption of pre-immune serum with any coagulase-positive strain, but not with coagulase-negative variants or L-forms.

Each unabsorbed antiserum produced bright fluorescence of all coagulase-positive strains and weak fluorescence of the coagulase-negative variants and L-forms.

Absorption of an antiserum with the homologous staphylococcus eliminated fluorescence of all staphylococci and L-forms tested. Use of coagulase-negative variant cocci as absorbing agents had no effect on the ability of an antiserum to produce fluorescence of typical *Staphylococcus aureus*, although the reaction of the encapsulated, coagulase-positive, clumping factor-negative (sv and κ6) cocci was somewhat weakened. The fluorescent reaction of typical *S. aureus* was diminished and that of coagulase-negative variants and L-forms was eliminated by absorption of antisera with cocci of strains sv or κ6. Surprisingly, absorption with sv or κ6 cocci only slightly decreased the effectiveness of *S. aureus* antisera in producing fluorescence of these encapsulated cells.

Table 3. *Effect of absorption of Staphylococcus aureus strain J17 antiserum with clumping factor-negative variants and L-forms of S. aureus on indirect fluorescent-antibody reactions*

Degree of fluorescence								
<i>S. aureus</i> tested (strain)	Pre-immune serum	Unabsorbed immune serum	Antiserum absorbed with					L-form of immunizing strain (J17)
			Cocci (strain)					
			J17	J17M	604M	SV	K6	
C+/CF+ *								
J17	+ †	+++	—	+++	+++	++	++	+++
604	+	+++	—	+++	+++	++	++	+++
2095	+	+++	—	+++	+++	++	++	+++
2253	+	+++	—	+++	+++	++	++	+++
s	+	+++	—	+++	+++	++	++	+++
C+/CF—								
SV	+	+++	—	++	++	++	++	+++
K6	+	+++	—	++	++	++	++	+++
C—/CF—								
J17M	—	+	—	—	—	—	—	+
604M	—	+	—	—	—	—	—	+
L-form								
J17	—	+	—	+	+	—	—	—

* C, coagulase reaction; CF, clumping factor activity.

† Degrees of fluorescence: + + +, Strong; + +, moderate; +, weak; —, negligible or none.

DISCUSSION

Variants of *Staphylococcus aureus* which are negative for both coagulase and clumping factor apparently possess few, if any, surface antigens characteristic of typical coagulase-positive staphylococci. These staphylococci were not agglutinated by antisera prepared against typical *S. aureus*. Absorption of anti-*S. aureus* sera with the cocci of coagulase-negative variants caused no significant decrease in *S. aureus* agglutination or clumping inhibition titres. It has been reported that the agglutinating antibody content of sera from rabbits immunized with coagulase-negative variants is remarkably low (Rotter & Kelly, 1966). Loss of protein A (antigen A), as well as of clumping factor antigen, accompanies coagulase-negative variation, as demonstrated by immunodiffusion studies (Blackstock & Kelly, 1968). Protein A has been recognized as a major agglutinin of *S. aureus* (Mudd, Yoshida, Li & Lenhart, 1963).

The encapsulated variants tested (sv and κ6) evidently have cell-wall antigens in

common with those of typical *Staphylococcus aureus* strains. Following absorption of *S. aureus* antisera with either sv or κ6 cocci, homologous agglutination titres were drastically decreased. Partial removal of clumping-inhibiting antibodies by sv and κ6 was an unexpected finding. Although they do not clump in the presence of fibrinogen, these staphylococci apparently have the antigen associated with the clumping reaction. The same conclusion was reached by Blackstock (1968), who found that antisera prepared against such encapsulated staphylococci contained high titres of clumping-inhibiting antibodies. Their failure to clump with fibrinogen could be due to a non-specific masking effect of the capsule. Another possibility is that a factor essential to cell-fibrinogen interaction may be separate from, though closely associated with, the antigen which functions in the clumping inhibition reaction (Kato & Omori, 1959). Recently, it was shown that the capsular polysaccharide of the coagulase-positive, clumping factor-negative staphylococci inhibited the fibrinogen precipitating activity of extracts of *S. aureus* (Blackstock *et al.* 1968). However, inhibition of clumping by this polysaccharide was not demonstrated.

L-forms of typical *Staphylococcus aureus* strains were no more effective in removing agglutinins and clumping-inhibiting antibodies than were coagulase negative, clumping factor-negative variants. A single absorption with the homologous L-form caused no change in titre. A second L-form absorption produced the results shown in Tables 1 and 2, i.e. a slight decrease in both agglutination and clumping-inhibition titres probably due to dilution of the antisera. Upon centrifugation of broth cultures, L-form growth was deposited in a viscous sediment with sufficient water content to account for the dilution.

Immunodiffusion experiments indicated that the L-forms liberated one or more antigens which were shared with the immunizing *Staphylococcus aureus* and with the encapsulated variants, but not with the coagulase-negative staphylococci. Free coagulase could be one such antigen. From results of preliminary studies, it appeared that clumping factor was not released into the medium by the L-form. Concentrated supernatant fluid of L-form broth cultures did not interfere with the clumping-inhibiting antibody activity of antisera.

Certain results of the fluorescent-antibody tests are noteworthy. Although pre-immune rabbit sera contained antibodies which caused slight fluorescence of coagulase-positive staphylococci, these sera failed to react with the coagulase-negative variants or the L-forms. Immunization with *Staphylococcus aureus* induced antibodies which gave weak fluorescence with both coagulase-negative variants and L-forms. Identical antigens have been found in extracts of the membranes of the coccal form and the L-form of *S. aureus* (Pratt, 1966). Absorption of antisera with either encapsulated or nonencapsulated, coagulase-positive staphylococci eliminated the fluorescent reaction of L-forms. Coagulase-negative variants were unable to remove the antibody responsible for fluorescence of L-forms.

Evidence that the L-form does not possess clumping factor can be found in results of the clumping-inhibition and fluorescent-antibody tests. L-forms did not remove clumping-inhibiting antibodies from *Staphylococcus aureus* antiserum. By use of the encapsulated staphylococci (sv and κ6) as absorbing antigens, antisera with agglutinin titres of 1/10 or less and clumping inhibition titres in the range of 1/80 to 1/640 were prepared. Such antisera did not cause fluorescence of the L-forms.

Application of the fluorescent-antibody technique to L-forms was most successful

when growth was taken from broth or soft agar cultures. Entire colonies, easily separated from the soft agar medium, were particularly suitable for these studies.

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