

# Role of Protein A in the Serum-Soft Agar Technique

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Formation of compact colonies of *Staphylococcus aureus* in serum-soft agar is mainly a result of a reaction between protein A and the Fc-part of immunoglobulin G and not a clumping factor-fibrinogen reaction.

*Staphylococcus aureus* cultivated in serum-soft agar produces two morphologically distinct colony types, compact and diffuse (3). The serum-soft agar technique has been suggested as a means of screening for encapsulated *S. aureus* strains, since encapsulated strains grow in the diffuse manner (21-25, 28, 29). The factor in rabbit serum responsible for producing compact colonies has not been investigated in detail, but compactness has generally been attributed to a reaction between the clumping factor and degradation products of fibrinogen (14, 21).

It is now well known that *S. aureus* producing protein reacts with the F-part of immunoglobulin G (IgG) in normal sera (4, 5, 7). This paper presents evidence that a protein A-Fc reaction can be detected when nonencapsulated *S. aureus* strains are grown in serum-soft agar and that this reaction is mainly responsible for the compacting action of normal serum in soft agar.

## MATERIALS AND METHODS

**Strains.** *S. aureus* types Cowan I, Smith diffuse, Smith compact, Scott strain "M" (NCTC 10649) (18), and Newman D2C were used. *S. aureus* Newman D2C was obtained from J. Hawiger, Vanderbilt University. The mutant of *S. aureus* type Cowan I lacking protein A (EMS 252) used in this report has been described earlier (6). One hundred *S. aureus* strains consecutively isolated in the routine laboratory were also used.

**Soft agar technique.** Soft agar medium was prepared by the method of Finkelstein and Sulkin (3). To 10 ml of this medium 0.1 ml of serum or a serum factor listed below was added. Bacteria to be cultivated in soft agar were grown in nutrient broth including 10% inactivated horse serum for 18 hr at 37 C. The bacteria were diluted 1:10<sup>4</sup> in 0.15 M NaCl, and 0.05 ml of this suspension was added to the tubes containing 10 ml of soft agar and incubated for 18 hr at 37 C.

Incorporated into the soft agar medium were the following. (i) Pooled serum from 10 nonimmunized rabbits. (ii) Rabbit IgG (10 mg/ml) purified by ammonium sulfate precipitation followed by chromatography on a diethylaminoethyl (DEAE)-cellulose column (8). Immunoelectrophoresis by the

method of Scheidegger (17) against donkey anti-rabbit plasma protein serum (Behring Werke AG) gave a single line corresponding to IgG. (iii) Rabbit immunoglobulin M (IgM) (1.0 mg/ml) purified on Sephadex G-200 followed by gradient elution from a DEAE-cellulose column as previously described (5). (iv) F(ab')<sub>2</sub>-fragments of rabbit IgG (6.6 mg/ml) were obtained by pepsin digestion by the method of Nisonoff (4, 15). (v) Fc-fragments of rabbit IgG (3.3 mg/ml) obtained by papain digestion by the method of Porter (5, 16). (vi) Human myeloma IgG (10 mg/ml) prepared by chromatography on DEAE-Sephadex (19). (vii) Human fibrinogen (Kabi grade L) (3 mg/ml) and doubling dilutions thereof. (viii) Rabbit fibrinogen (3 mg/ml) (Koch Light Labs) and doubling dilutions thereof.

**Capsule staining.** Capsule staining was performed in India ink preparations (2).

**Heat treatment.** Heat treatment of serum was performed at 60 C for 10 or 30 min to inactivate degradation products of fibrinogen (14).

**Agglutination.** Agglutination and testing for clumping were performed on slides using 40  $\mu$ liters of human serum, immunoglobulin, or fibrinogen solution in appropriate dilutions. Smears were made with a platinum loop and the reaction was recorded as positive if definite agglutination or clumping occurred within 5 min. All experiments included controls for spontaneous agglutination, performed in 0.15 M NaCl (5).

**Determination of protein A.** Protein A was extracted from *S. aureus* by the method of Jensen (12). The extract was diluted approximately 1:1 in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, so that 1 ml of the final solution of crude protein A corresponded to 5 ml of broth culture of *S. aureus* containing 10<sup>9</sup> colony-forming units/ml. The hemagglutination technique for quantitation of protein A (20) was applied to doubling dilutions of the material to be tested. The concentrations of protein A were determined by reference to a standard preparation of heat-extracted cell-bound protein A obtained from *S. aureus*, Cowan I, and highly purified by DEAE-Sephadex chromatography and gel filtration (9).

## RESULTS

**Morphology of *S. aureus* in soft agar including serum or fibrinogen.** Ninety-two of 100 *S. aureus*

strains consecutively isolated in the routine department and subcultured approximately 10 times showed compact morphology in serum-soft agar with rabbit normal serum; eight were diffuse. When heat-treated serum was used, the strains formed compact and diffuse colonies as in the presence of normal serum. A high percentage of strains (84%) also showed compact morphology in soft agar containing fibrinogen in a concentration of 3 mg/ml. However, soft agar containing fibrinogen in lower concentrations showed a lower compacting capacity. When fibrinogen was added in a concentration of 0.4 mg/ml or less, only diffuse colonies were formed for all strains tested. The levels of fibrinogen degradation products in normal human serum are even lower: 0.6 to 2.6  $\mu\text{g/ml}$  (11, 13). Rabbits are known to have a very low fibrinolytic activity (10), with resulting low levels of fibrinogen degradation products. The results show that the compact morphology of *S. aureus* in serum-soft agar is not due to a clumping effect of fibrinogen, as the level of fibrinogen equivalents was too low.

**Morphology of *S. aureus* in soft agar containing IgG and IgM.** Table 1 lists the morphology of *S. aureus* in the presence of rabbit IgG, fragments thereof, and rabbit IgM. A high percentage of strains (86%) showed compact morphology in soft agar containing IgG and F-fragments of IgG. No compaction was observed in soft agar containing F(ab')<sub>2</sub>-fragments of IgG or IgM. Approximately the same number of strains formed compact colonies in the presence of human myeloma IgG as in rabbit serum.

Table 2 shows the morphology of the investigated strains in soft agar containing IgG in relation to the protein A content of the strains. Strains with very low or undetectable protein A grew diffusely, strains with a moderate production of protein A mainly formed compact colonies, and strains with a high production of protein A grew compactly. Of strains with

TABLE 1. Colony morphology of 100 *S. aureus* strains in soft agar containing rabbit serum, rabbit IgG, Fc- or F(ab')<sub>2</sub>-fragments of rabbit IgG, or rabbit IgM

| Soft agar containing:     | Compact | Diffuse |
|---------------------------|---------|---------|
| Rabbit serum.....         | 92      | 8       |
| Rabbit IgG.....           | 86      | 14      |
| Fragments of rabbit IgG   |         |         |
| Fc.....                   | 86      | 14      |
| F(ab') <sub>2</sub> ..... | 0       | 100     |
| Rabbit IgM.....           | 0       | 100     |

diffuse growth, two were shown to be encapsulated in India ink preparations. One of them produced a moderate amount of protein A.

Table 3 shows the morphology in soft agar containing normal rabbit IgG or F(ab')<sub>2</sub>- or F-fragments of IgG of five *S. aureus* strains, all previously well characterized. *S. aureus*, Cowan I, with a high protein A production showed compact morphology in soft agar containing IgG or Fc-fragments of IgG but not with F(ab')<sub>2</sub>-fragments of IgG. The mutant of Cowan I without any detectable protein A or capsule showed diffuse morphology.

The nonencapsulated Smith variant with a high production of protein A showed compact morphology in the presence of IgG or Fc but not with F(ab')<sub>2</sub>. The encapsulated Smith

TABLE 2. Colony morphology of 100 *S. aureus* strains in soft agar containing rabbit IgG in relation to their protein A content

| Protein A content (ng/10 <sup>9</sup> bacteria) | No. of strains |         |
|---|----------------|---------|
|   | Compact        | Diffuse |
| <47   | 0              | 12      |
| 47-375  | 41             | 3       |
| 750-3,000                                       | 44             | 0       |

TABLE 3. Colony morphology of three *S. aureus* strains and their variants in soft agar containing rabbit IgG, Fc- or F(ab')<sub>2</sub>-fragments of rabbit IgG (protein A content is indicated in the table)

| <i>S. aureus</i> strain           | Morphology <sup>a</sup> with: |    |                     | Protein A content (ng/10 <sup>9</sup> bacteria) |
|-----------------------------------|-------------------------------|----|---------------------|---|
|                                   | Rabbit IgG                    | Fc | F(ab') <sub>2</sub> |   |
| Cowan I,<br>Wild type             | C                             | C  | D                   | 1,500-3,000                                     |
|                                   | D                             | D  | D                   |   |
| Protein A-negative mutant         |                               |    |                     | <47   |
| Smith,<br>Encapsulated variant    | D                             | D  | D                   | 1,500-3,000                                     |
| Nonencapsulated variant           | C                             | C  | D                   | 1,500-3,000                                     |
| Scott,<br>Strain "M" encapsulated | D                             | D  | D                   | 1,500-3,000                                     |

<sup>a</sup> C, Compact morphology; D, diffuse morphology.

variant and the Scott encapsulated strain with a high protein A content grew diffusely in soft agar containing all serum components.

The results in Tables 2 and 3 clearly show that the compacting action of serum in soft agar is mainly due to a protein A-Fc reaction and that diffuse colonies are formed by encapsulated *S. aureus* strains and also by strains with no or very low protein A production.

**S. aureus strain Newman D2C slide test with fibrinogen and IgG.** *S. aureus*, type Newman D2C, mainly used in the so-called staphylococcal clumping test for quantitation of human fibrinogen and its degradation products, proved to have a relatively high content of protein A: 750 to 3,000 ng/10<sup>9</sup> bacteria. When this strain was tested on slides in dilutions of human IgG or fibrinogen, agglutination and clumping, respectively, occurred with both at a dilution approximately 1:40 of the normal human serum level.

Thus the protein A-IgG reaction may interfere with the staphylococcal clumping test for fibrinogen, giving falsely positive or falsely high values. However, in sera with elevated levels of fibrinogen split products, the protein A-IgG reaction probably is of minor importance. نظمت

#### DISCUSSION

Finkelstein and Sulkin (3) reported that staphylococci grown in soft agar containing normal human or rabbit plasma or serum form characteristic colonies. Coagulase-positive staphylococci grow as compact colonies whereas coagulase-negative staphylococci grow as diffuse colonies. The factor in serum and plasma giving compact growth was shown to be associated with the globulin fraction of serum and to be stable at 56 C for 30 min. Serum from rabbits immunized with coagulase-positive strains of staphylococci was shown to have a higher capacity to produce compact colonies. The conclusion was that human and rabbit sera contain antibody-like factors capable of altering the colony morphology of most *S. aureus* strains.

Alami and Kelly (1) tested *S. aureus* in soft agar containing human or rabbit serum or plasma and found that 80% of *S. aureus* strains showed compact morphology. When 0.4% bovine fibrinogen was included in the soft agar, approximately the same percentage of compact colonies was formed. They concluded that the reaction in fibrinogen-soft agar is an expression of staphylococcal clumping factor. However, they found that plasma and serum are more effective than fibrinogen alone in producing compact staphylococcal colonies. They discussed

a possible role of normally occurring antibodies in the compacting action of normal serum. Lipinski et al. (14) and Hawiger et al. (11) showed that fibrin lysates produced by plasmin digestion clumped *S. aureus* on slides and that fibrin monomer complexes within the lysate were responsible for the clumping reaction. Heating at 60 C for 10 min precipitated this fibrin monomer complex, thereby destroying the substrate for clumping factor. However, a reaction with immunoglobulins was not investigated.

In a recent series of papers, K. Yoshida and co-workers (21-29) investigated encapsulation of *S. aureus* by the serum-soft agar technique. They suggested that fibrinogen or its degradation products produce compact colonies in serum soft agar (21). However, the experiments reported in this paper clearly show that a reaction between protein A and the Fc-part of IgG is mainly responsible for the formation of compact colonies in soft agar. More than 95% of staphylococcal strains producing high or moderate amounts of protein A formed compact colonies in serum-soft agar. The same result was obtained whether rabbit IgG or Fc-fragments of the globulin were incorporated in the soft agar. Strains producing little or no protein A formed diffuse colonies only with IgG or Fc. Six strains with compact growth in serum-soft agar were diffuse in soft agar including only IgG or Fc-fragments. The discrepancy could be due to additional factors giving compact colonies in serum or due to altered reactivity with IgG in the preparation procedure. However, encapsulated strains of *S. aureus* producing high or moderate amounts of protein A also gave only diffuse colonies. The implication is that in encapsulated strains a reaction between protein A and IgG does not occur, probably because protein A is covered by a surface capsule. Yoshida and co-workers (26) also found that some *S. epidermidis* strains grew with compact morphology in serum-soft agar. However, we have observed that some *S. epidermidis* and *Micrococcus* strains show compact morphology when grown in the basal medium without serum incorporated (U. Forsum and E. Hjelm, unpublished observations). No such growth was observed with *S. aureus*.

Our experiments show that when fibrinogen is present in high concentrations in soft agar, compact colonies of *S. aureus* are formed, but that when fibrinogen is present in concentrations equivalent to normal serum concentrations, only diffuse colonies are formed. The experiments also show that, when the staphylococcal clumping test on slides is used for quantitation of concentrations of fibrinogen or its degradation products,

the protein A-IgG reaction can be misinterpreted as a clumping reaction.

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