CHARACTERISTICS OF COAGULASE POSITIVE AND COAGULASE NEGATIVE STAPHYLOCOCCI IN SERUM-SOFT AGAR[1]

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Preliminary experiments revealed that coagulase positive staphylococci could be distinguished culturally from coagulase negative staphylococci in a soft agar medium containing a small amount of normal human or rabbit plasma. In a medium of suitably low viscosity, staphylococci produce elongated diffuse feathery colonies. When coagulable plasma is incorporated in such a medium, the colonies of coagulase positive staphylococci are compact and spherical, whereas colonies of coagulase negative staphylococci are essentially unaltered. It was considered, at this point, that coagulase elaborated by microcolonies affected the plasma resulting in increased viscosity in the immediate colonial environment which, in turn, influences the morphology of the developing colony. Although this premise may be untenable, some observations made in an attempt to elucidate the mechanism of this phenomenon are of interest and may find application in studies of staphylococci and other bacteria.

The use of soft or semisolid agar media in the differentiation of microorganisms is not unique. Pike (1946) used a modification of the soft agar medium of Ward and Rudd (1938) in differentiating streptococci. Morphological changes of colonies in a semisolid medium were used as an indication of pneumococcus transformations (McCarty *et al.*, 1946). This technique was later applied by Ravin (1954). Pittman and Davis (1950) used growth characteristics in semifluid media to distinguish species of *Haemophilus*. A double layer technique with antiserum-containing soft agar was utilized by Stocker (1949) in studies on flagellar antigenic phases in Salmonella. Knox *et al.* (1956) recently reported on characteristics of growth of *Mycobacterium tuberculosis* in semisolid agar media. Lankford *et al.* (1955) successfully used soft agar, with and without antiserum, for demonstrating antigenic variation through morphological differences of colonies of *Vibrio cholerae*. Also, the ability of flagellar antiserum to inhibit swarming in soft agar has been used in the identification of *Salmonella* species (Cooper, 1956).

MATERIALS AND METHODS

Most of the strains of staphylococci used were isolated from patients in Parkland Memorial Hospital. Dr. Betty Hobbs graciously supplied strains representative of Cowan's serotypes (Cowan, 1939) together with small amounts of the corresponding antisera (Hobbs, 1948). Coagulase reactions were verified by both the tube and slide techniques (Cadness-Graves *et al.*, 1943). Phage type and other characteristics were determined by Dr. Eugene Rosenblum. Stock cultures initiated from isolated colonies were maintained on trypticase soy agar. The soft agar basal medium contained (in per cent) tryptone (Difco), 1.0; yeast extract (Difco), 0.5; glucose, 0.5; K_2HPO_4, 0.5; agar (Difco), 0.15; in distilled water. After the medium was sterilized by autoclaving and cooled to 50 C, the desired amounts of sterile plasma or serum were added. Ten ml of medium were pipetted into tubes previously seeded with 0.05 ml of a 10^{-6} dilution of a suspension in 5 ml of the 18 hr slant growth of the strain to be tested. This was found to give a suitable number of suspended isolated colonies from most strains in 18 hr at 37 C. The tubes may be layered with 1 ml of 2 per cent agar which helps prevent the formation of an aqueous condensate in which growth may occur. The medium is too fluid to be used as a plating medium. Antisera were prepared by immunization of rabbits with heat-killed washed cells according to the technique described by Hobbs (1948), and sera were adsorbed with heat-killed and/or living cells.

RESULTS

In preliminary experiments with a series of 50 staphylococcus cultures, coagulase producing strains were readily distinguished when 1.0 per

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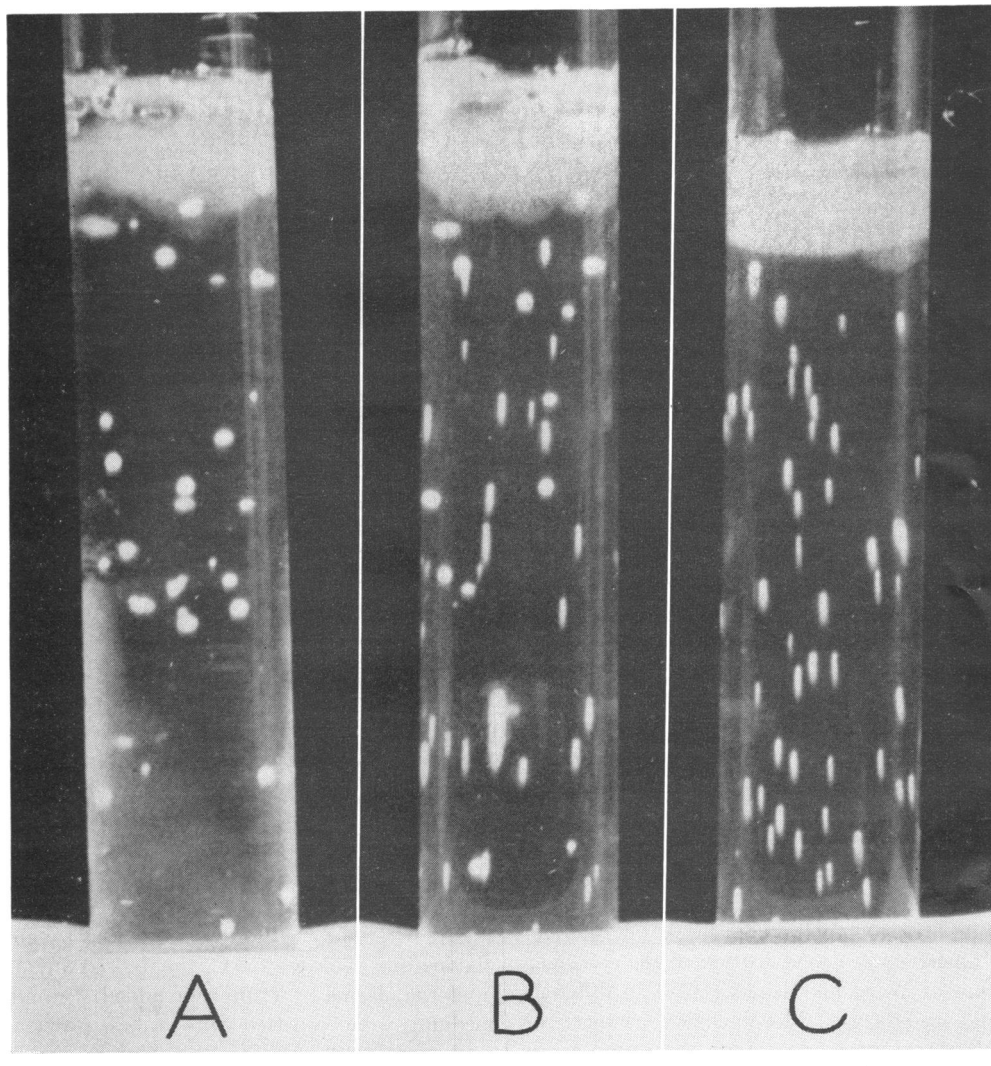

Figure 1. Comparison of colony morphology of coagulase positive (*A*) and negative (*C*) staphylococci, and artificial mixture (*B*) in soft agar containing normal human plasma (1:100). After incubation at 37 C for 24 hr.

cent normal human or rabbit plasma was incorporated in the soft agar basal medium. All strains produced diffuse colonies in the unsupplemented medium. In plasma media, the coagulase positive strains produced compact spherical colonies, while growth of coagulase negative strains was essentially the same as in the control medium (figure 1). To gain more information regarding the mechanism of the reaction, serum from the donors (humans and rabbits) was tested. Serum, which is not coagulated by coagulase, should have no effect if the "compacting" mechanism depends on coagulase activity. The response to serum was identical to that of plasma.

The "colony compacting factor" of normal serum and plasma appears to have certain of the properties generally attributed to antibody. The activity is associated with the globulin fraction of serum precipitated by half-saturation with $(NH_4)_2SO_4$, is stable on heating at 56 C for 30 min and is removed following adsorption with cells of coagulase positive strains. In addition, the colony compacting titer of rabbit serum rises during immunization with coagulase posi-

TABLE 1

Effect of immunization of rabbits with heat-killed coagulase positive Staphylococci (strain J) on colony compacting and agglutinin titers

Number of Injections*	Days After Initial Injection	Agglutinin Titer*	Colony Compacting Titer
0	1	64†	1,000‡
3	14	1024	10,000
7	33	4096	30,000
8	58	4096	30,000

* Antigen: heat-killed (60 C/1 hr) suspension of washed staphylococci.

† Reciprocal of highest serum dilution causing agglutination of suspension of heat-killed cells. Incubation: 2 hr at 50 C followed by overnight at 4 C.

‡ Reciprocal of highest serum dilution causing definite alteration of colony morphology in soft agar incubated 18 hr at 37 C.

tive strains with accompanying increases in agglutinin titer (table 1). However, the colony compacting effect is manifest at higher serum dilutions than is agglutination.

A variety of colony forms may be observed in serum media dependent on such factors as the concentration and potency of the serum and the time of incubation. In relatively high serum concentrations, the spherical compact colony type is formed. If lower serum concentrations are used or incubation is prolonged in moderately high serum concentrations, the colonies exhibit slight tail formation (figure 2) which becomes more marked with time or further serum dilution. When serum is diluted beyond the effective concentration, colonies are diffuse and feathery similar to those in the control medium. Concentrations of agar higher than 0.2 per cent tend to make the colonies more compact thus decreasing the visible effect of serum. Occasionally halos are seen around compact, spherical colonies in media containing high concentrations of serum.

Coagulase negative strains vary somewhat in their response to these conditions. Particularly, variation has been noted in the ability of strains to grow in the deeper portion of the medium. Colonies may be well developed throughout the tube, or they may develop toward the upper surface and remain barely visible or absent in the deep portions. These characteristics may be related in part to oxygen requirements (Evans *et al.*, 1955). Plasma or serum may be slightly stimulatory or slightly inhibitory to growth. In no case among the strains examined have colonies of coagulase negative strains shown any similarity to the compact spherical type of coagulase positive colony observed in serum media.

To determine if the soft agar technique could be rendered serologically type-specific, specific immune sera were prepared by immunization of rabbits with strains representative of the Roman numeral serotypes of Cowan (1939). Since these sera showed considerable cross-reactivity, they were exhaustively adsorbed with heterologous types (table 2). It can be seen that although there is some degree of cross-reaction between types I and III, the colony compacting phenomenon can be rendered type specific by use of appropriate dilutions of specific immune sera.

An additional staphylococcus culture (strain Smith) was found by Hunt (1957, *personal com-*

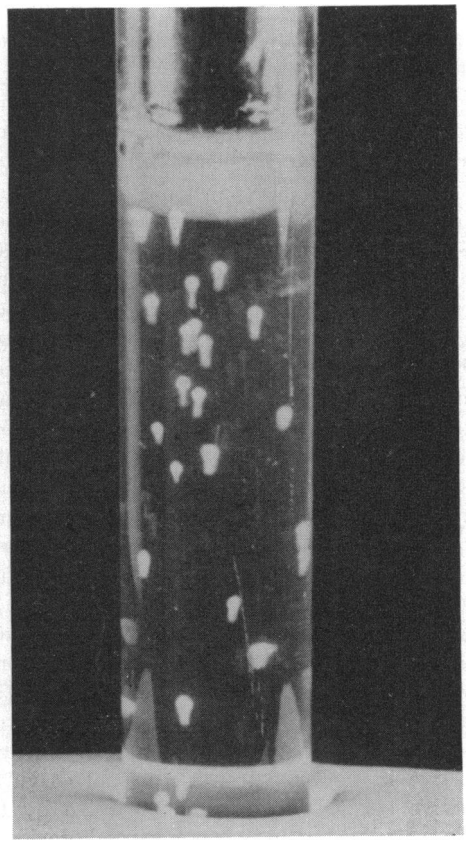

Figure 2. Tailed colonies of coagulase positive staphylococci in soft agar containing rabbit antiserum (1:1000) after incubation at 37 C for 40 hr. The "tails" developed after 18 hr incubation.

TABLE 2

Specificity of colony compacting activity of adsorbed anti-staphylococcal serum

Immune Serum	Adsorbed With (Types)	Colony Compacting Activity				
		Test strain	Serum dilution			No serum
			1:100	1:300	1:1000	
I	II, III	8530	++++†	++++	++++	−
		8531	+	±	−	−
		8532	+++	++	+	−
II	I, III	8530	−	−	−	
		8531	++++	+++	++	
		8532	−	−	−	
III	I, II	8530	−	−	−	
		8531	−	−	−	
		8532	+++	+	±	

* Staphylococcus strain 8530 (type I); 8531 (type II); 8532 (type III).

† Degree of colony compactness: − = diffuse colony; ++++ = compact spherical colony.

munication) to consist of a mixture of diffuse and compact colony types when tested with a sample of normal human plasma in the soft agar technique. Both types were coagulase positive. These observations were confirmed with a transplant of this culture provided by Dr. Hunt, and substrains were established for further tests by picking representative colonies from serum-soft agar tubes with sterile Pasteur pipettes. The original mixture agglutinated in type II serum, was sensitive to phage 44A (Blair and Carr, 1953), and was coagulase positive by both slide and tube tests. The predominant component of the mixture, which produced compact colonies in normal serum and type II antiserum, reacted similarly. However, substrains established from diffuse colonies in normal or type II serum failed to agglutinate in types I, II, or III sera and were resistant to phage 44A and all others commonly used in phage typing of coagulase positive staphylococci (Blair and Carr, 1953). They were also negative by the slide coagulase test but positive in the tube test. Occasional coagulase positive strains which are nonreactive by the slide coagulase test have been reported previously (Cadness-Graves *et al.*, 1943). Variants could also be distinguished in the original "mixed" culture by subtle colonial differences when examined by oblique transmitted light (Henry, 1933) on trypticase soy agar plates.

To determine whether these variants originated spontaneously, pure cultures of each type were established by three successive streak platings of isolated colonies. Infusion broth was inoculated with the purified substrains and examined at daily intervals for evidence of variation using the soft agar technique with type II serum and by plating on trypticase soy agar. After incubation for approximately 120 hr, some evidence of heterogeneity became apparent in the culture of the diffuse (inagglutinable, phage insensitive) substrain and two types of variants were isolated. One produced mucoid colonies on trypticase soy agar. It was inagglutinable, slide coagulase positive and yielded few discrete plaques with the routine test dilution of phage 44A. The other variant, which was not mucoid, was agglutinated by type II serum, lysed confluently by the routine test dilution of phage 44A, and was positive in the slide coagulase test. This type could not be distinguished from the original compact colony form. The compact type is more stable in broth but, on prolonged incubation yields a few diffuse type variants detectable by the serum-soft agar technique. The patterns of sensitivity of all variants, diffuse, compact, and mucoid, to 11 antibiotics[2] and triple sulfa were identical using the disc technique. Polymyxin B was the only drug to which they were resistant. The sensitivity patterns further tend to substantiate the endogenous origin of the variants which if examined individually might be considered to have sufficient differences to be classed as distinct strains (Williams and Rippon, 1952). All the variants were hemolytic on rabbit blood agar and were capable of splitting phenolphthalein phosphate (Barber and Kuper, 1951).

DISCUSSION

Apparently, many normal human and rabbit sera contain antibodylike factors capable of altering the colony morphology of most strains of coagulase positive staphylococci cultivated in soft agar media. This observation provides a technique for distinguishing colonies of coagulase

[2] Aureomycin, bacitracin, carbomycin, chloromycetin, dihydro-streptomycin, erythromycin, neomycin, penicillin, terramycin, tetracycline and polymyxin B.

positive and negative strains. The significance and origin of these antibodylike factors remains to be evaluated. They may be a consequence of previous exposure to coagulase positive (virulent) strains. However, laboratory rabbits were not found to be nasal carriers of coagulase positive staphylococci (Rountree *et al.*, 1956) and might acquire this antibodylike factor by means other than through natural infection. Perhaps of significance is the observation that germ free chickens can produce agglutinins in response to dead micrococci administered in the diet (Wagner, 1955).

The mechanism of formation of compact colonies in specific serum-soft agar may depend on the interaction between certain heat-stable antigens situated at or near the surface of the cell (Oeding, 1953) and specific antibody resulting in adherence (agglutination) of daughter cells during the course of development of the colony.

The demonstration that the colony compacting reaction can be made serologically specific by the use of immune serum, in lieu of normal serum or plasma, should make this technique a useful tool in studies of the dynamics of coagulase and antigenic variation of staphylococci.

The technique was applied in experiments with an unusual staphylococcus strain which yielded two types of colonies on primary examination by the serum-soft agar technique. Although the substrains derived from the two colony forms were distinguishable by serological reaction, phage sensitivity, slide coagulase reaction, and subtle colonial differences revealed with oblique light, it was demonstrated that these variations can arise in pure cultures by spontaneous mutation. Although these variants may have been detected by laborious selection and testing of isolated colonies, the serum-soft agar technique provides the advantage of simultaneous isolation and serological determination of large numbers of colonies. In addition, this technique appears to provide a more sensitive indication of antibody content of sera than conventional agglutination reactions possibly because only single cells and small clones participate in the reaction rather than heavy suspensions of organisms.

Modification of the technique to suit the requirements and characteristics of the organism being studied may prove useful in studies of antigenic variation in other bacteria.

SUMMARY

Normal rabbit and human plasma and sera have been shown to contain a factor(s) resembling antibody which alters the colonial morphology of coagulase positive staphylococci in a soft agar medium. This observation has been applied in a technique which permits the differentiation of colonies of coagulase positive and coagulase negative staphylococci. When specific immune serum is used in lieu of normal serum or plasma, colonies of heterologous antigenic type can readily be distinguished from the compact colonies of the homologous type.

REFERENCES

BARBER, M. AND KUPER, S. W. A. 1951 Identification of *Staphylococcus pyogenes* by the phosphatase reaction. J. Pathol. Bacteriol., **63,** 65–68.

BLAIR, J. E. AND CARR, M. 1953 The bacteriophage typing of staphylococci. J. Infectious Diseases, **93,** 1–13.

CADNESS-GRAVES, B., WILLIAMS, R., HARPER, G. J., AND MILES, A. A. 1943 Slide test for coagulase positive staphylococci. Lancet, **1,** 736–738.

COOPER, G. N. 1956 A rapid method for the identification of *Salmonella* species. J. Pathol. Bacteriol., **72,** 39–45.

COWAN, S. T. 1939 Classification of staphylococci by slide agglutination. J. Pathol. Bacteriol., **48,** 169–173.

EVANS, J. B., BRADFORD, W. L., JR., AND NIVEN, C. F., JR. 1955 Comments concerning the taxonomy of the genera *Micrococcus* and *Staphylococcus*. Intern. Bull. Bacteriol. Nomen. and Taxon., **5,** 61–66.

FINKELSTEIN, R. A. AND SULKIN, S. E. 1957 Colonial agglutination of coagulase positive staphylococci in soft agar. Bacteriol. Proc., **1957,** 97–98.

HENRY, B. S. 1933 Dissociation in the genus *Brucella*. J. Infectious Diseases, **52,** 374–402.

HOBBS, B. C. 1948 A study of serological type differentiation of *Staphylococcus pyogenes*. J. Hyg., **46,** 222–238.

KNOX, R., SWAIT, E., AND WOODROFFE, R. 1956 The growth of *Mycobacterium tuberculosis* in semi-solid agar media. J. Gen. Microbiol., **15,** 359–371.

LANKFORD, C. E., EKLUND, C. E., AND ALAMI, S. Y. 1955. Methods for investigation of variation in somatic antigens of *Vibrio cholerae*. Bacteriol. Proc., **1955,** 88.

MCCARTY, M., TAYLOR, H. E., AND AVERY, O. T.

1946 Biochemical studies of environmental factors essential in transformation of pneumococcal types. Cold Spring Harbor Symposia Quant. Biol., **11**, 177–183.

OEDING, P. 1953 Serological typing of staphylococci. II. Investigations on the serological, chemical and physical properties of the antigens. Acta Pathol. Microbiol. Scand., **33**, 312–323.

PIKE, R. M. 1946 A study of group A streptococci from healthy carriers with particular attention to mucoid polysaccharide production. J. Infectious Diseases, **79**, 148–156.

PITTMAN, M. AND DAVIS, D. J. 1950 Identification of the Koch-Weeks bacillus (*Hemophilus aegyptius*). J. Bacteriol., **59**, 413–426.

RAVIN, A. W. 1954 A quantitative study of autogenic and allogenic transformations in pneumococcus. Exptl. Cell Research, **7**, 58–82.

ROUNTREE, P. M., FREEMAN, B. M., AND JOHNSON, K. G. 1956 Nasal carriage of *Staphylococcus aureus* by various domestic and laboratory animals. J. Pathol. Bacteriol., **72**, 319–321.

STOCKER, B. A. D. 1949 Measurements of rate of mutation of flagellar antigenic phase in *Salmonella typhimurium*. J. Hyg., **47**, 398–413.

WAGNER, M. 1955 Germfree research: a basic study in host-contaminant relationship. II. Serologic observations in germfree animals. Bull. N. Y. Acad. Med., **31**, 236–239.

WARD, H. K. AND RUDD, G. V. 1938 Studies on hemolytic streptococci from human sources. I. The cultural characteristics of potentially virulent strains. Austral. J. Exptl. Biol. Med. Sci., **16**, 181–192.

WILLIAMS, R. E. O. AND RIPPON, J. E. 1952 Bacteriophage typing of *Staphylococcus aureus* J. Hyg., **50**, 320–353.