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THE IMMUNIZING PROPERTIES OF A *STAPHYLOCOCCUS AUREUS* VARIANT POSSESSING SURFACE ANTIGEN

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INTRODUCTION

The controversy as to whether the antitoxin or antibacterial antibodies are responsible for immunity to infection by *Staphylococcus aureus*, has raged for many years (Cameron, 1963). In reviewing the relevant literature Koenig *et al.* (1962 b) concludes that " These studies show that although deaths produced by ordinarily lethal doses of alpha haemolysin can be prevented by immunization with crude alpha haemolysin toxoid, only heat killed staphylococcal vaccine protects mice against death following infection with viable replicating staphylococci ".

New light was thrown on the problem of staphylococcal immunity when Dubos isolated the so-called Smith strain from a case of osteomyelitis in man in 1930 (Morse, 1965). This strain differs from typical virulent *S. aureus* in the following respects:

1. It has an exceptionally high pathogenicity for mice. The LD50 is $37 \cdot 5 \times 10^6$ organisms (Ekstead, 1963a). The pathogenicity is due to resistance to phagocytosis (Morse, 1960, 1962; Koenig, 1962), and can be greatly enhanced by mucin (Fisher, 1960).

2. It produces free coagulase but no bound coagulase (clumping factor), and grows as diffuse colonies in soft serum agar. The colonial morphology has been found to be closely related to the presence of bound coagulase (Finkelstein & Sulkin, 1958; Alami & Kelly, 1959; Takamasa *et al.*, 1963). According to these findings it should be possible to identify strains which possess surface antigen by simply testing free coagulase and determining bound coagulase or colony morphology. Fahlberg (1962), however, found that free and bound coagulase and capsular material are unrelated.

3. Smith strains have been found to possess exceptional immunizing properties (Koenig, 1962; Koenig *et al.*, 1962a; Tompsett, 1962; Ekstead, 1963a; Lauria & Kaminski 1963). This immunizing ability has been shown to be due to a surface antigen (Fisher, 1960) which is also immunogenic in purified form (Morse, 1962; Fisher *et al.*, 1963). It is heat stable (Fisher, 1960), will precipitate specifically with Smith antiserum (Morse, 1962, 1963) but does not produce precipitating antibody (Morse, 1962).

Immunization with surface antigen results in an increase in the phagocytic index (Tompsett, 1962; Lauria & Kaminski, 1963). By means of phagocytic tests it has been shown that both a heat-stable serum factor resembling antibody and a heat-labile factor resembling complement were required for opsonization (Cohn & Morse, 1959; Koenig *et al.*, 1962a; Li *et al.*, 1963). Ekstead (1963a) found that the ability of sera to enhance the phagocytic power of normal mouse leucocytes showed a correlation with the protective properties of the sera. These results stress the importance of surface antigen as an immunizing agent.

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The somatic antigens are also probably important in producing immunity and may be strain specific (Greenberg & Cooper, 1961; Greenberg *et al.*, 1961; Juergens *et al.*, 1963; Ekstead, 1963b, 1963c; Stamp, 1964; Stamp & Edwards 1964). Bearing this in mind a large number of bovine strains isolated from milk and udder skin were examined for the presence of surface antigen. No such strain could, however, be found.

Smith strains are prone to variation (Lenhardt *et al.*, 1961; Greenberg, 1963), and as Wiley (1961) and Fahlberg (1962) were able to produce encapsulated organisms, the possibility of producing an encapsulated variant from a bovine strain was investigated. An attempt was also made to assess the relative immunizing importance of toxoid, surface antigen, and somatic antigens against infection with living organisms.

Materials and Methods

Strains

Two Smith type strains were obtained, one from Prof. R. Ekstead[1] (Ekstead, 1963a), and one from Dr. M. G. Koenig[2] (Koenig, 1962) and an encapsulated strain was received from Dr. B. Wiley[3] (Wiley, 1961). The Cowan Types I, II, III and Wood 46 were acquired from National Collection of Type Cultures, Colindale, London.

Strains 24710, 2553, 10979, 11391 and strain 24276, from which the variant was produced, were isolated from acute cases of bovine mastitis. Strain 24276 was free- and bound-coagulase positive, phosphatase positive, egg yolk factor positive, fermented mannitol anaerobically and produced both alpha and beta haemolysin. It is therefore a typical pathogenic strain, but produced diffuse colonies in serum soft agar. The other strains were typical, pathogenic *S. aureus*.

Strain T48T was isolated from the udder skin of a cow. Its biochemical reactions were typical for *S. aureus*.

Media

The following media were employed during the course of this study.

Bicarbonate agar (Burbon & Wende, 1960), Worfel-Ferguson agar (Edwards & Ewing, 1955), glycerol broth (Wiley, 1961), semi-synthetic broth (Wiley, 1961), Pertussis agar (Mason, 1965), glycero-phosphate broth and agar (Pattison & Mathews, 1957), Zierdt agar (Zierdt *et al.*, 1963), heart brain infusion agar (Difco), trypticase soy agar (T.S.A.) (National Biochemical Co.), soft serum agar (Finkelstein & Sulkin, 1958) and mannitol salt agar (Chapman, 1945).

Biochemical and serological tests

The biochemical characteristics of the strains were determined by the methods described by Baird-Parker (1963).

Alpha and beta haemolysin production was tested in 0·5 per cent nutrient agar at pH 7·8 under 30 per cent CO_2 (Cameron, 1965).

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Colony morphology was examined by inoculating screwcapped tubes of soft serum agar with 0·5 ml of a 10^{-8} saline dilution of an overnight broth culture. The tubes were gently shaken and examined after 24 hours incubation at 37° C.

Slide agglutination and agglutinin absorption tests were done according to the procedure described by Oeding (1957).

Micro-precipitin tests were done on microscope slides covered with 5·0 ml 1·0 per cent Ion-agar (Difco) in distilled water. Sodium azide (0·5 per cent) was incorporated in the agar to control contamination. Wells, 2·0 mm diameter and 4·0 mm apart, were made in the agar by means of a small cork bore and perspex template. One drop of reagent was placed in each well, the slides placed in petri dishes with a disc of moist blotting paper, and left in an incubator at 37° C overnight. The slides were examined in oblique light against a dark background for the presence of precipitin lines. This method gave excellent results.

Antiserum was prepared in rabbits with 24 hour glycerophosphate broth cultures killed by the addition of 0·5 per cent formalin. The antigen was administered intravenously according to the schedule of Wiley (1961).

Surface antigen was extracted with 5 ml 0·1 N acetic acid from cells derived from cultures grown overnight in glycero-phosphate agar contained in Mason tubes (Fisher *et al.*, 1963). These methods of antiserum and antigen preparation were experimentally shown to give the best results

Production and examination of variants

The strains examined (Cowan I, Cowan II, Cowan III, 24276, 24710 and T. 48T) were inoculated into 10 ml semi-synthetic broth in 1-ounce McCartney bottles in triplicate and incubated at 37° C. Subinoculations of 0·5 ml from these cultures were made into similar bottles with the same medium every 24 hours, 48 hours, 7 days and 30 days depending on the different experiments.

Simultaneously with each passage in semi-synthetic broth, a loopful of culture from each bottle was plated out on mannitol salt agar to obtain single colonies. After 48 hours incubation of the plates at 37° C, six single colonies were subcultured onto glycero-phosphate agar in tubes. These cultures were used to inoculate glycero-phosphate agar, nutrient broth and nutrient agar in Mason tubes for the determination of surface antigen, colony morphology, and free and bound coagulase respectively.

Preparation of vaccine and immunity tests

Cellular vaccine was prepared from cells grown on glycero-phosphate agar or broth. The cells were suspended in saline, and the density adjusted according to the requirements of the different experiments. The vaccines were inactivated with 0·5 per cent formalin or by autoclaving.

Toxoid was prepared by formalizing toxin produced in 0·5 per cent heart brain infusion agar (Difco) pH 7·8. Alpha toxin was produced from Wood 46 (haemolytic titre 1/1000) and beta toxin from strain 2553 (haemolytic titre 1/800) (Cameron, 1965).

Surface antigen was prepared by extracting the cells obtained from one glycero-phosphate agar Roux flask culture with 25 ml 0·1N acetic acid (Fisher *et al.*, 1963).

Combined vaccines containing either toxoid plus cells or surface antigen plus cells were prepared by suspending the desired number of cells in either toxoid or surface antigen.

All experimental animals were immunized by two injections of vaccine with an interval of three weeks. The routes and doses varied with the different experiments.

Fifty adult mice and 25 guinea pigs per group were used in all the experiments.

Ten days after the second injection the immunized animals were challenged with 0·2 ml of a saline suspension of strain 24276 containing 4,000 $\times$ 10^6 cells per ml. Mice were challenged intraperitoneally and guinea pigs subcutaneously. Controls were challenged simultaneously.

The protective value of the different vaccines for mice was determined by calculating the percentage survivors 48 hours after challenge. The guinea pigs were slaughtered and examined six days after challenge and figures of 4 +, 3 +, 2 +, 1 + and 0 allocated to each animal according to the extent and severity of the lesions. The controls were similarly assessed and the difference between the control and immunized groups was expressed as the percentage protection.

Results

Antiserum prepared from cells grown in glycero-phosphate broth gave the highest precipitating titres while the highest concentration of surface antigen was obtained when cells grown on the same medium were extracted with 0·1N acetic acid (Fisher *et al.*, 1963).

Monthly and weekly passage of strains in semi-synthetic broth did not give rise to Smith type variants. Strain 24276 showed the greatest tendency to variation and one colony was found to be free coagulase positive and bound coagulase negative. It produced diffuse colonies in soft serum agar but did not contain surface antigen. Other variants were found which were bound coagulase positive but produced diffuse colonies and vice versa.

These findings prompted an experiment designed to determine the validity of the soft serum agar test as an indication of the presence or absence of bound coagulase and to find whether the colony compacting effect is due to bound coagulase or some serum factor. The results obtained showed that colony morphology is not dependent on bound coagulase or surface antigen.

After the failure to induce Smith type variants by monthly and weekly passage, an extensive experiment was undertaken in which three stains were passaged in semi-synthetic broth every 48 hours for eight generations. A large variety of variants were obtained, but there did not appear to be any orderly variation pattern.

At the sixth passage single colonies from strain 24276 and Cowan I were found giving positive precipitin reactions with Smith type antiserum, but such variants were not found again at the 7th and 8th passage.

A similar experiment in which passage was carried out every 24 hours also yielded a number of variants, but none which reacted with Smith type antiserum.

Variant No. 1 from passage 6 of strain 24276 gave the clearest precipitin line and was used for further experiments. It was designated 24276 (65VI).

With the aim of increasing the number of single colonies, extracts of which would react with Smith type antiserum, this variant was plated out repeatedly on various media generally used to promote the production of capsular material. None, however, increased the number of single colonies giving positive precipitin reactions, and as the clearest reactions were obtained with extracts of cells grown on glycero-phosphate agar, this medium was used throughout subsequently.

Strain 24276 (65VI) was now plated out on glycero-phosphate agar, twelve single colonies tested for surface antigen and the one giving the clearest precipitin line subjected to further selection. After four selections all single colonies contained surface antigen. This characteristic remained constant on further subculturing.

Variant 5 from generation 4 was designated 24276 (68V5), freeze-dried, and used for further experiments.

To show the difference among them, the more important biochemical characteristics of strains 24276 (original), 24276 (65VI), 24276 (68V5) and two typical Smith strains are compared in Table 1.

TABLE 1.—*Characteristcs of typical Smith strains and variants of strain* 24276

Strain	Free coagulase	Bound coagulase (Clumping factor)	Colony morphology (Finkelstein serum agar)	Average alpha haemolysin titre	Average beta haemolysin titre
24276 (original).	+	+	Diffuse	9	213·0
24276 (65VI)...	+	+	Compact	21·2	7·0
24276 (68V5)...	+	+	Compact	1·6	0
Smith (Ekstead)	+	−	Diffuse	6·6	0
Smith (Koenig).	+	−	Mixed	n.t.	n.t.

+ = Positive; − = Negative; n.t. = Not tested

Variant 68V5 of strain 24276 differs from the parent strain in that it has lost its ability to produce haemolysin. However, it remained bound coagulase positive and also produced compact colonies in soft serum agar.

It differs from classical Smith strains because it is bound coagulase positive, grows as compact colonies in soft serum agar and is not pathogenic for mice. All of ten mice given 760×10^6 organisms intraperitoneally survived.

In order to confirm that the surface antigen obtained from strain 24276 (68V5) is identical to the Smith surface antigen, antiserum was prepared in rabbits and cross precipitin tests carried out, the results of which are shown in Table 2 and Plate 1.

TABLE. 2—*Cross precipitin reaction of strains* 24276 (*original*) *and* 24276 (68V5) *with Smith type strains*

Surface antigen extracts from strains	Rabbit antisera from strains			
	Smith (Ekstead)	Smith (Koenig)	24276 (68V5)	Wiley
Smith Ekstead.....	+	+	++	−
Smith Koenig.....	+	+	++	−
24276 (68V5).....	+	+	++	−
24276 (original).....	−	−	−	−
Wiley.....	−	−	−	−

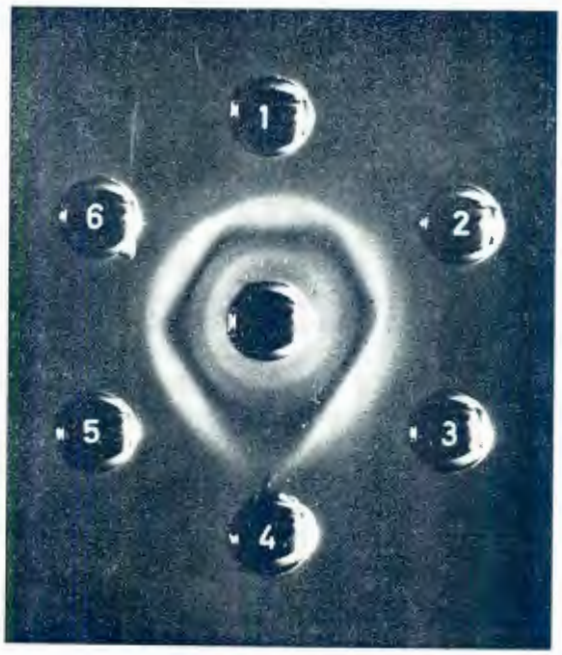

PLATE 1.—Precipitin reactions of surface antigen from Smith strains and strain 24276 (68V5 with antiserum to strain 24276 (68V5)
Centre well—24276 (68V5) antiserum.

Well 1 Surface antigen from Smith Strain (Ekstead).

Well 2 Surface antigen from Smith Strain (Koenig).

Well 3 Surface antigen from strain 24276 (68V5).

Well 4 Extract of strain 24276 (original).

Well 5 Surface antigen from 24276 (65VI).

Well 6 Surface antigen from 24276 (68V5).

The convergent lines produced by extracts of strains Smith (Ekstead), Smith Koenig and 24276 (68V5) against antiserum from strain 24276 (68V5) confirm the identity of the antigens. It is also evident that the original strain contains no surface antigen and it appears that the Wiley strain, if it is indeed capsulated, has no serological relationship with Smith type surface antigen.

The immunizing value of formalin killed fluid cultures containing 116×10^8 and 120×10^8 organisms per ml respectively of strains 24276 (orginal) and 24276 (68V5) were compared. There was no significant difference between the two strains. Neither could any significant discrepancy between the routes of immunization be found, which is contrary to the results obtained by Lauria *et al.* (1963).

The protection afforded by both these vaccines was rather poor, and an experiment was therefore conducted to determine the minimal immunizing dose. According to the results obtained with vaccines produced on glycero-phosphate agar as shown in Table 3, at least 50×10^8 organisms are required to produce a good immunity.

TABLE 3.—*Determination of minimal immunizing dose of strain* 24276 (68V5) *for mice by the intraperitoneal route*

Vaccine		Total mice challenged	Total survivors	Overall Protection %	Protection -controls %
Cells per ml $\times 10^8$	Dose ml				
320	0·3	48	44	91·6	67·6
320	0·1	48	39	81·2	57·2
160	0·3	47	42	89·3	65·3
160	0·1	49	34	69·4	45·4
80	0·3	49	36	73·5	49·5
80	0·1	47	24	51·1	27·1
30	0·3	48	24	50·0	26·0
30	0·1	46	16	34·7	10·7
Controls	—	50	12	24·0	0·0

NOTE.—Mice were challenged intraperitoneally

A further experiment was conducted to determine whether the surface antigen had any immunizing properties or whether the immunity obtained was solely due to somatic antigen. It was also designed to determine whether autoclaving would have any effect on the immunizing properties of whole cells.

From the results in Table 4 it can be deduced that, although surface antigen from strain 24276 (68V5) has considerable immunizing properties, it does not materially add to the immunizing power of washed cells devoid of surface antigen. It also appears that the immunizing ability of the cells is not affected by autoclaving.

Toxoid was also tested, but gave virtually no protection.

A similar, but less extensive, experiment was carried out in guinea pigs. The results shown in Table 5 were virtually identical to those obtained in mice, except that toxoid had a reasonably protective effect. It must, however, be pointed out that the lesions produced were not purulent, but rather a necrotic phlegmosis. Where the toxic effects of the infection predominate, it can be expected that antitoxic immunity will reduce the symptoms.

TABLE 4.—*Evaluation of immunizing properties of surface antigen cells, and toxoid for mice*

Vaccine					Total mice challenged	Total survivors	Overall protection %	Protection-controls %
Composition		Treatment of cells	Cells killed by	Density cells/ml × 10^8				
Surface antigen	Cells strain							
+	—	—	—	—	50	41	82·0	44·0
+	24276 (68V5)	None	0·5% Formalin	320	44	41	93·2	55·2
+	24276 (orig.)	None	0·5% Formalin	292	48	46	95·8	57·8
None	24276 (68V5)	None	0·5 Formalin	320	48	45	93·7	55·7
None	24276 (orig.)	None	0·5% Formalin	292	50	48	96·0	58·0
None	24276 (68V5)	Extracted with 0·1N acetic acid	Autoclaved	320	50	45	90·0	52·0
None	24276 (orig.)	do	do	292	45	41	91·1	53·1
	Toxoid	—	—	—	50	22	44·0	6·0
	Controls	—	—	—	50	19	38·0	0·0

NOTE.—Mice were immunized by two intraperitoneal injections of 0·2 ml each and also challenged intraperitoneally

TABLE 5.—*Comparison of immunizing properties of cells, surface antigen and toxoid for guinea pigs*

Vaccine		Total animals examined	Possible positive units	Positive	Negative	Overall protection %	Protection -controls %
Composition	Dose ml						
24276 (68V5) cells..... (320 × 110^8 cells/ml)	0·5	23	92	32	60	65·4	44·4
24276 (orig.)..... (292 × 10^8 cells/ml)	0·5	22	88	31	57	64·9	43·9
Surface antigen.....	0·5	23	92	40	52	56·5	35·5
Toxoid.....	0·5	21	84	35	49	58·3	37·3
Controls.....	—	25	100	79	21	21·0	0·0

s.c. = Subcutaneously

NOTE.—Guinea-pigs were immunized and challenged subcutaneously

Conclusions

Normally pathogenic strains of *Staphylococcus aureus* were shown to give rise to variants possessing surface antigen when passaged in semi-synthetic broth. The appearance of such variants was sporadic and no clear variation pattern according to other characteristics was evident.

The presence of surface antigen was not related to bound coagulase or colony morphology which is in accordance with the findings of Fahlberg (1962). The colony compacting phenomenon seen in soft serum agar did not appear to be solely due to bound coagulase but was also dependent on a serum factor (other than agglutinin) which could be removed by absorption.

Not all media were suitable for the production of surface antigen. In this study glycero-phosphate agar gave the best results.

The variant produced differs from the classical Smith strains in that it produces bound coagulase, grows as compact colonies in soft serum agar and has a low pathogenicity for mice. The surface antigen from it is, however, serologically identical to Smith surface antigen (S.S.A.).

The chemical analysis of S.S.A. (Morse, 1962; Perkins, 1963) and staphylococcal polysaccharide antigen (S.P.A.) (Fisher *et al.*, 1963; Haskell & Henessian, 1963) is very similar to that of capsule material from Wiley's strain (Wiley & Wonnacott, 1962). No cross precipitin reactions could, however, be demonstrated and it appears that Wiley's strain is not capsulated. This is strongly supported by recent work by Mudd & DeCourcy (1965).

The surface antigen extractable from strain 24276 (68V5) is immunogenic but the strain itself does not possess exceptional immunizing properties.

Discussion

Obtaining suitable experimental animals is one of the major obstacles in assessing the immunizing role of different antigens to staphylococcal infections. Mice have a high natural resistance, intradermal infection in rabbits results in necrosis rather than abscessation and subcutaneous lesions do not form consistently in guinea pigs. It is difficult to reproduce natural purulent infections in laboratory animals. The lesions are usually of toxic origin, which is not the case in natural infection. This fact may explain why such good results have been obtained in the past by immunization with toxoid alone (Downie, 1937; Forsman, 1937, 1938). It may be possible to overcome the inability to produce purulent infections in laboratory animals by introducing organisms subcutaneously on silk sutures (MacLeod *et al.*, 1963).

The value of different immunizing antigens such as toxoid, surface antigen and coagulase may or may not depend on the pathogenic mechanisms of the specific strain (Alami & Kelly, 1960; Li, 1962; Fisher, 1963; Smith, 1962, 1963; Forster, 1963; Lam *et al.*, 1963b; Taubler *et al.*, 1963; Borchardt & Pierce, 1964). The results presented in this paper, however, stress the immunizing importance of somatic antigens. This is in accordance with the results recently published by Stamp (1964) and Stamp & Edwards (1964) who were able to immunize rabbits with a protein fraction. It also agrees with the findings of Higgenbotham & Bass (1961), Fisher (1963) and Lam *et al.* (1963a) who showed that staphylococci possess somatic aggressins.

The work of Ekstead (1963b, 1963c) indicates that there are at least two immunogenic protein fractions, one protecting against organisms suspended in mucin, but not saline, and a heat labile fraction protecting against organisms suspended in saline.

The results obtained in this laboratory showed that there was no appreciable deterioration in immunogenicity of autoclaved cells when the mice were challenged with saline suspended organisms. It therefore appears that the overall role of Ekstead's heat labile protein is small and that a third fraction possibly also plays a role in conferring immunity.

As with the extracellular antigens, it is probable that no single somatic antigen is responsible for immunity.

Summary

A *Staphylococcus aureus* variant was produced from a typical pathogenic strain by passage in semi-synthetic broth. It possesses surface antigen serologically identical to Smith surface antigen, but the variant differs from typical Smith strains in that it is bound coagulase positive, forms compact colonies in soft serum agar and is avirulent for mice.

The surface antigen is immunogenic, but immunity experiments stressed the importance of the protective properties of somatic antigens.

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