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EVALUATION OF SEROLOGICAL TESTS AS CRITERIA FOR IMMUNITY TO STAPHYLOCOCCAL SKIN INFECTION IN RABBITS

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Abstract

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Rabbits immunized with killed whole culture vaccine, bacteria alone or bacteria plus toxoid, were markedly more resistant to skin infection than control rabbits. However, the degree of immunity was not related to the antitoxin or haemagglutinating titres, nor to the opsonizing activity of their sera.

Introduction

Despite the mass of available data, the nature and mechanism of resistance to staphylococcal infections is by no means well understood (Ekstedt, 1965). One of the major problems is the absence of a reliable *in vitro* assay method which would be a direct and meaningful measure for the immune status of an immunized animal. In order to resolve this problem, numerous authors have attempted to isolate antigens in pure form which would induce a protective immunity. If such an antigen could be identified it could be used in serological tests, which would then be a direct measure for actual resistance to infection. The literature pertaining to this field of investigation has been reviewed in previous publications and it is apparent that no such antigen has as yet been found (Cameron, 1963; Cameron, 1969).

It has been shown that when hyperimmune serum is absorbed with cell wall teichoic acid, the opsonizing potency of the serum is markedly reduced (Mudd, Yoshida, Li & Lenhart, 1963; Cameron, 1969), but other antigens may exhibit similar properties. Ekstedt (1966) also presented evidence that teichoic acid is an important antigen for inducing immunity. These findings appeared to have promise and it was therefore desirable to investigate the immunizing properties of teichoic acid more thoroughly.

The majority of experimental work done with staphylococci has been conducted in mice and the criterion for immunity has been survival or death after intraperitoneal challenge. This is a convenient but unsatisfactory model because, as has been pointed out previously, the mechanism of immunity in an acute infection may be different from the mechanism involved in a localized chronic infection (Cameron, 1969). The latter is the usual form of staphylococcal infection and it was therefore decided to use abscess formation in the skin of rabbits as the experimental model. This model has also been used successfully by other authors (Greenberg & Cooper, 1961).

Assay of the immunizing properties of purified teichoic acid poses two problems. Firstly, it is not antigenic in purified form and its immunological properties cannot be studied by active immunization of experimental animals with pure material. It was therefore decided to study the production of anti-teichoic acid antibodies in animals immunized with whole bacteria and to correlate these results with actual resistance to infection.

The second problem was to find a satisfactory method to assay anti-teichoic acid antibodies. The haemagglutination test is one of the most sensitive and was the method of choice. Pure teichoic acid, however, does not sensitize tanned erythrocytes and consequently cannot be used in this form (Oeding, Grov & Myklestad, 1964; Grov, 1965a; Daugherty, Shriver & White, 1967; Yoshida & Ekstedt, 1968). An impure polysaccharide (Polysaccharide A) was therefore employed. It contains primarily teichoic acid (Davidson, Baddiley, Hofstad, Losnegard & Oeding, 1964), but also possesses a mucopeptide moiety which is responsible for its ability to sensitize erythrocytes (Grov, 1965b).

As stated earlier, antibodies to teichoic acid promote phagocytosis and this phenomenon is widely accepted as an important factor in rendering animals resistant to infection. It was therefore also deemed necessary to determine whether any correlation could be found between the opsonizing activity of serum and immunity.

Antitoxins have been shown to play only a minor part in protection to infection with living bacteria (Koenig, Melly & Rogers, 1962; Stamp, 1964; Cameron, 1966). The strain used in these studies, however, produces a large amount of beta toxin and, because antibodies to beta toxin may be important in this particular case, their role was also examined in one experiment.

Finally, the strain specificity of immunity to staphylococcus is an unresolved question and some experiments were done in both rabbits and mice in an attempt to clarify this problem. Stamp (1964), Stamp & Edwards (1964), Angyal (1966) and Hill (1969) contend that immunity is not type specific, while Greenberg & Cooper (1960) and Warner, Slipetz & Kroeker (1966) are of the opposite opinion.

Materials and Methods

Strains

Staphylococcus aureus (Rosenbach, 1884) strain 24276 was isolated from a case of bovine mastitis. It is coagulase positive and produces primarily beta haemolysin as judged by the pattern of haemolysis on blood tryptose agar plates. The strain was found to be composed primarily of light colonies and a small number of slightly pigmented colonies. The culture was cloned and the light colonies designated 24276(w) and the others 24276(G). Clone 24276(w) was used throughout. *S. aureus* 68V5 is a mutant of strain 24276 and is of the Smith compact type. Both these strains were described in detail by Cameron (1966). *S. aureus* (Smith) was obtained from Dr. R. D. Ekstedt* and the Wood 46 strain from Dr. R. K. Lindorfer**. With the exception of strain 4769, which was isolated from a case of canine endometritis, all the other strains used were isolated from cases of acute bovine mastitis.

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Preparation of vaccines

The vaccines used were either formalized bacterial suspensions, cell suspensions mixed with toxoid or formalized whole cultures. Bacteria were grown on P & M broth (Pattison & Matthews, 1957) in agitated Roux flasks at 37°C for 24h and killed by the addition of 0,5 per cent formalin.

Beta toxin was produced from strain 4769 by a similar method to that described for alpha toxin production (Cameron, 1965). Briefly the procedure was to grow the organisms in 400 ml Brain Heart Infusion broth (Difco)* containing 0,5 per cent agar in penicillin flasks. The flasks were incubated for 48h at 37°C in air. Hereafter 200 ml 0,15 M phosphate buffer pH 7,2 was added to each flask. The flasks were then left at 4°C overnight and the extracted toxin cleared by centrifugation at 3 000*g* for 20 min. Haemolytic activity was assayed by adding 0,1 ml of a 10 per cent washed sheep red blood cell suspension to 1,0 ml volumes of twofold dilutions of toxin made in the same buffer as used for extraction. The tubes were incubated at 37°C for 60 min and subsequently at 4°C for 60 min. The end point was taken as the highest dilution showing complete haemolysis. The toxin was toxoided by the addition of 0,7 per cent formalin and detoxification was allowed to proceed at room temperature for 7 to 10 days.

All the vaccines used for rabbits contained 0,5 per cent packed bacteria in the final product, while the vaccines for mice contained 1,0 per cent packed cells. These figures correspond to approximately 1,5 and 3,0 mg dry bacteria per ml and are equivalent to about $2,5 \times 10^9$ and 5×10^9 bacteria per ml respectively.

When toxoids and adjuvants were incorporated into the vaccine, the bacterial density was increased to allow for the dilution. For alum precipitated vaccines the appropriate volume of a 12 per cent solution of potassium alum was added to give a final concentration of 2,0 per cent. In order to obtain maximal precipitation the pH of all alum adjuvant vaccines was adjusted to 4,5 with 7,5 per cent KOH.

Immunization and challenge of experimental animals

Female albino rabbits were immunized by either the intravenous or subcutaneous route. In the former case the vaccine was administered according to the schedule of Wiley (1961). The dose and schedule for the subcutaneous procedure was varied according to the requirements of each specific experiment. Groups of six rabbits were used for every vaccine.

The rabbits were challenged 2 weeks after the last dose of vaccine by the intradermal injection of live bacteria. Care was taken to perform all the injections in skin areas showing no active hair growth. Bacteria used for challenge were produced in P & M broth shake cultures incubated at 37°C for 18 to 24 h. The bacteria were deposited by centrifugation, resuspended in half the original volume of saline and 1:2, 1:5 and 1:10 dilutions prepared. The hair on one of the flanks of the rabbits was clipped on the previous day and 0,1 ml of the concentrated bacterial suspension as well as of the three dilutions was injected intradermally using a 24 gauge hypodermic needle. According to live counts done on the bacterial suspensions the numbers of live bacteria were in the range of 10^9, 5×10^8, 2×10^8 and 10^8 per 0,1 ml dose respectively.

The diameters of the lesions were measured 7 days later and the surface area calculated. The histograms represent the average surface area of the lesions at every dose level in each group of experimental animals.

Groups of ten 12-week-old male albino mice were used to assay each preparation. Every mouse received two subcutaneous injections of vaccine with an interval of 3 weeks and was challenged 2 weeks after the second dose by the intraperitoneal injection of live bacteria suspended in hog gastric mucin. The challenge procedure was based on the method described by Parker, Warner & Slipetz (1966). Bacteria were grown in P & M broth, collected by centrifugation and suspended in saline to give a density of approximately 8×10^8 live bacteria per ml. The density was determined by means of an Unigalvo nephelometer calibrated to give a reading of 80 on the linear scale with standard *Brucella* agglutination antigen. According to live counts a density reading of 10 corresponded to 8×10^8 live bacteria per ml. The undiluted suspension was used as well as 1:2 and 1:4 dilutions. One volume of bacterial suspension was mixed with two volumes of gastric mucin and 0,75 ml of the mixture injected intraperitoneally into mice. Each mouse thus received 0,25 ml of bacterial suspension representing challenge doses of approximately 2×10^8, 1×10^8, or $0,5 \times 10^8$ live bacteria. Deaths were recorded daily for 3 days.

Serological tests

Sera from immunized and non-immunized rabbits were obtained on the day prior to challenge and assayed by means of the haemagglutination test according to the method described by Morse (1962). Polysaccharide for sensitizing tanned sheep erythrocytes was prepared from *S. aureus* strain 24276 as described by Haukenes, Losnegard & Oeding (1961).

Beta antitoxin was assayed by a method similar to that described by Cruickshank (1965) for alpha antitoxin. Dilutions were made in phosphate buffer saline pH 7,3 and sheep erythrocytes were used instead of rabbit erythrocytes. The tubes were incubated at 37° for 60 min followed by cooling to 4°C for 60 min.

Phagocytosis

The procedure followed was essentially as described previously (Cameron, 1969). However, as it was desired to compare the opsonizing activity of different sera, they were inactivated at 56°C for 30 min and fresh guinea pig serum added as a source of complement. The final reaction mixture was composed as follows:

Leucocyte suspension ($2 - 5 \times 10^6$/ml)	2,0 ml
Bacterial suspension ($1 - 2 \times 10^7$/ml)	2,0 ml
Inactivated serum	0,5 ml
Guinea pig serum (Complement)	0,5 ml

In some experiments P & M medium did not give satisfactory bacterial growth. Consequently bacteria were produced in Brain Heart Infusion broth (Difco) and plate counts done on Trypticase Soy agar (BBL)*.

Results

Immunization of rabbits

It is well known that strains of *S. aureus* vary markedly in their pathogenicity for experimental animals depending on the types and quantity of toxins produced. Preliminary experiments were therefore done to find a strain which would consistently produce abscesses in the skin of rabbits. Of ten bovine strains examined, only one strain, 24276(w) produced purulent lesions. The majority of the strains gave large necrotic lesions while

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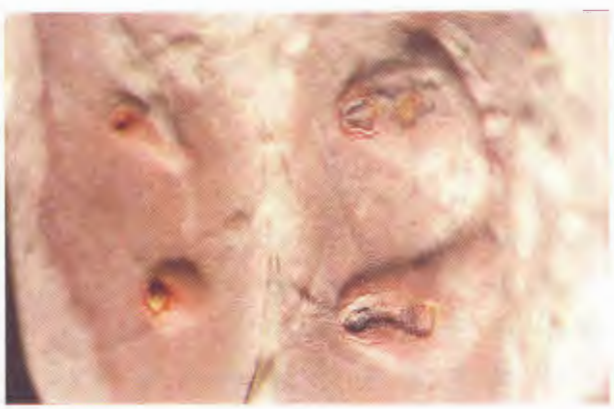

PLATE 1 Nature of lesions produced by *S. aureus* in the skin of immune (left) and normal rabbit (right).

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the others were of low virulence and intradermal injection of these strains resulted in very small and transient lesions. Typical abscesses produced by strain 24276(w) in an immunized and control animal are shown in Plate 1.

Figure 1 shows the results of an experiment in which different routes and schedules of immunization were compared. Groups of rabbits were immunized with a suspension of killed bacteria by either a series of intravenous or subcutaneous injections according to the schedule of Wiley (1961), while a third group received the same total amount of cell vaccine divided into four doses. There was very little difference in the degree of immunity among the three groups when compared with the controls. It is significant that the group immunized by intravenous injection had an average haemagglutination titre of 1:392, while the two groups immunized by the subcutaneous route had average titres of only 1:47 and 1:10 respectively in spite of showing the same degree of immunity. Wild & Rogers (1960) have also recorded similar high haemagglutination titres after intravenous immunization of rabbits. It is apparent that a high antibody titre is not required for protection and that repeated subcutaneous injections afford the best immunity.

However, protracted series of subcutaneous injections is cumbersome and other schedules were therefore compared. Either two or four injections of alum precipitated whole culture vaccine were given to groups of rabbits at 2 or 4 week intervals. According to the results presented in Fig. 2 there was no significant difference between the various schedules and consequently only two injections were given at an interval of 4 weeks in subsequent experiments.

The results presented in Fig. 3 show that vaccines composed of cells plus toxoid, cells alone or toxoid alone were equally effective in inducing a solid immunity.

Evaluation of serological tests as criteria for immunity

The haemagglutination titres of individual immunized rabbits plotted against the sum of the surface area of the lesions provoked after challenge is depicted in Fig. 4. The result shows a complete random distribution and there is obviously no direct correlation between haemagglutination titre and the degree of resistance to infection.

A similar comparison of beta antitoxin titres and immunity is shown in Fig. 5. Again there is no relationship between antitoxin titres and immunity.

In order to examine the role of opsonization in immunity, the sera of three normal rabbits were compared in three separate experiments with sera obtained from intravenously immunized rabbits and with sera from rabbits immunized by two subcutaneous injections of alum precipitated whole culture vaccine. The sera from the immunized rabbits were obtained from animals used in previous experiments and shown to be immune

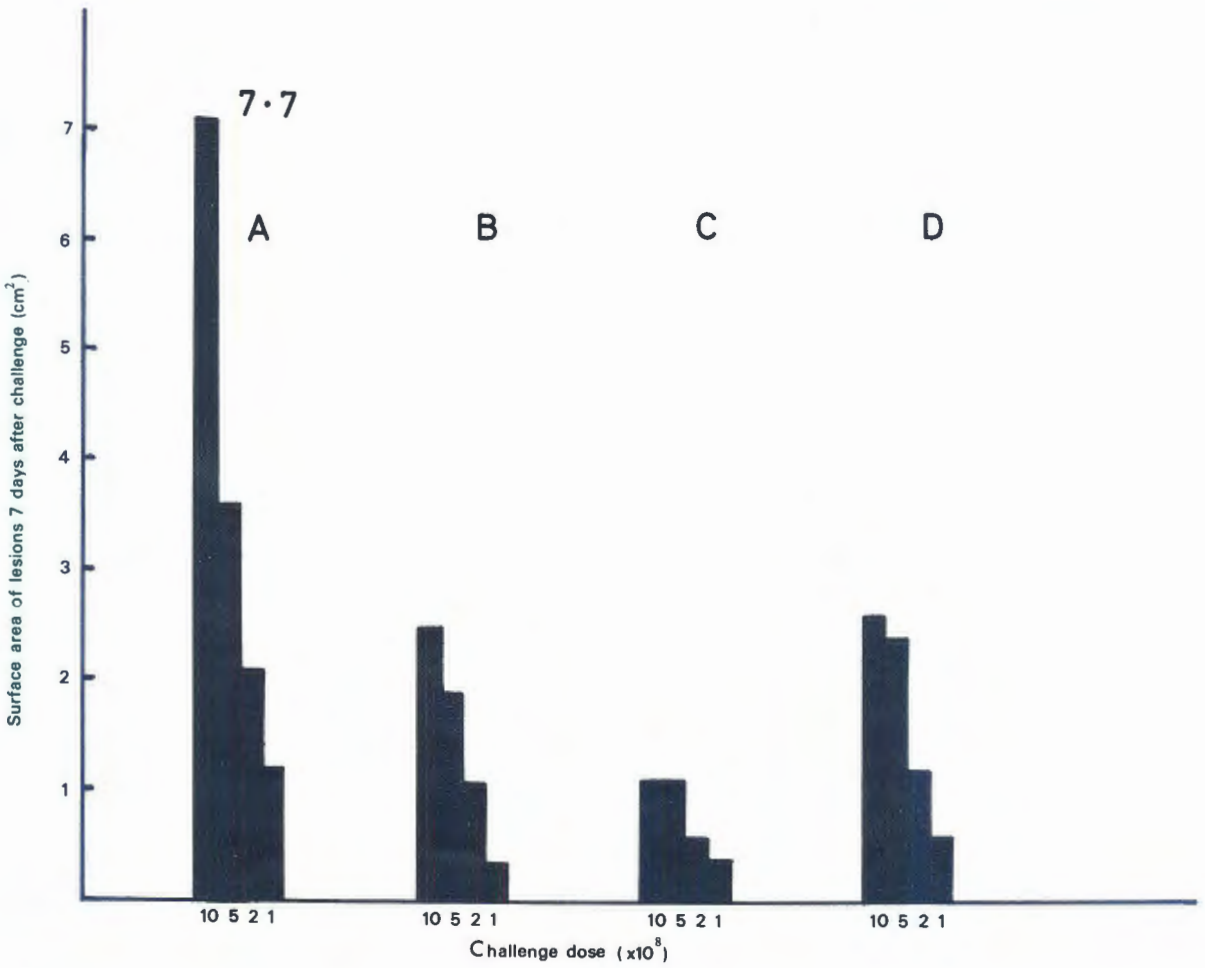

FIG. 1 Comparison of route and schedule of vaccine administration on immunity to skin infection in rabbits. A = Non-immunized controls; B = Series of intravenous injections; C = Series of subcutaneous injections; D = Two subcutaneous injections

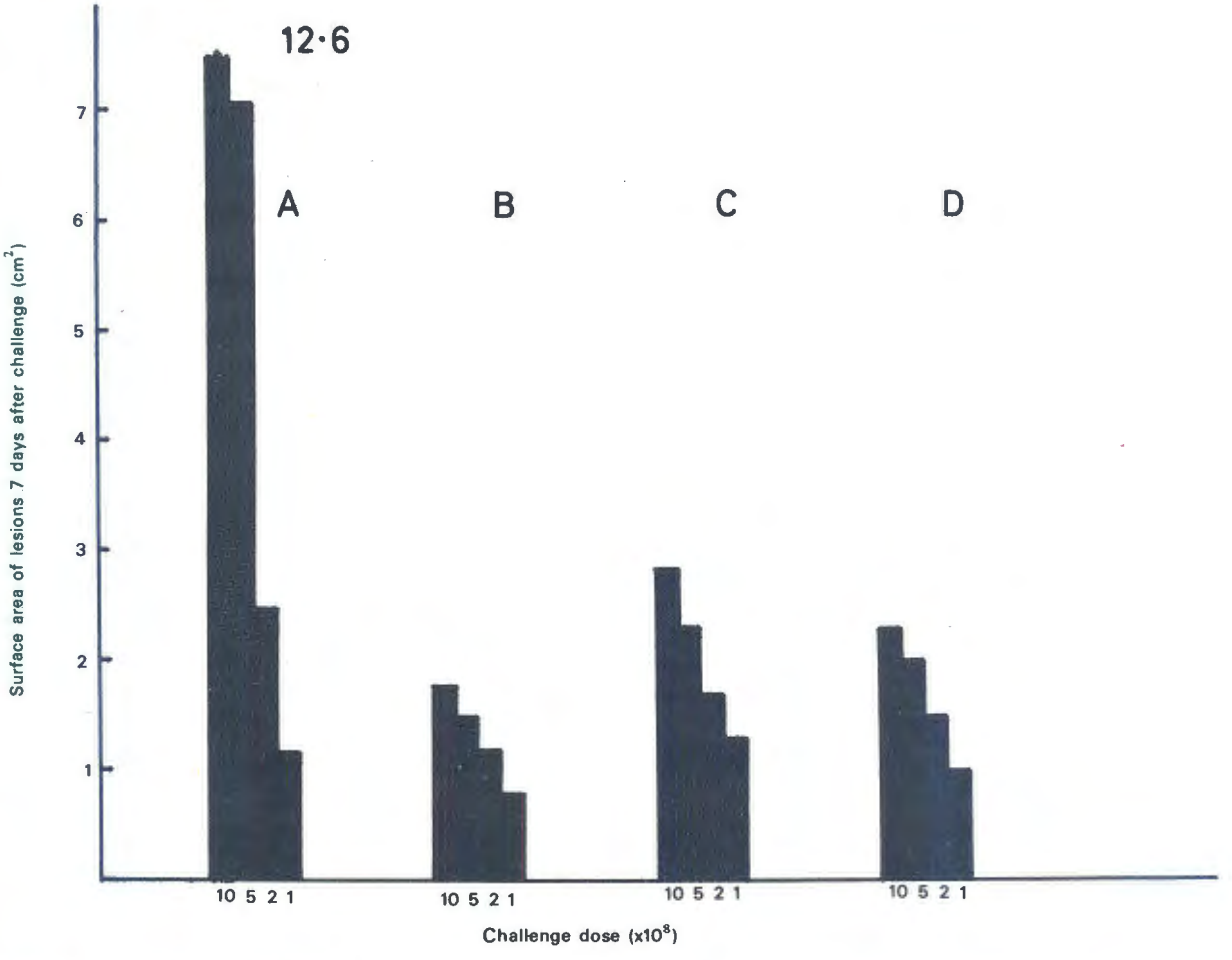

FIG. 2 Influence of immunization schedule with whole culture vaccine on immunity on skin infection in rabbits. A = Non-immunized controls; B = Four injections at two weeks intervals; C = Two injections with two weeks interval; D = Two injections with four weeks interval

to intradermal challenge with living staphylococci. The HA titres of these sera are shown in Table 1.

TABLE 1 *Haemagglutination titres of six immune rabbits immunized either subcutaneously or intravenously*

Serum No.	Route of immunization	HA titre
1	intravenous	1:640
2	,,	1:2 560
3	,,	1:10 240
4	Subcutaneous	1:32
5	,,	1:64
6	,,	1:64

The HA titres of the three sera from normal rabbits were 1:8, 1:8 and 1:4 respectively.

The results of the three experiments are shown in Fig. 6.

In the first experiment there was very little difference in the opsonizing activity of the three sera despite the marked differences in HA titres. The sera from the immune rabbits were in fact slightly less effective than the normal serum.

In the second experiment the activity of the serum from the intravenously immunized rabbit (HA titre 1:2 560) was essentially the same as that of the serum obtained from a normal rabbit (HA titre 1:8) while the activity of the serum from the subcutaneously immunized rabbit was the poorest.

In the third experiment normal rabbit serum was again the most potent while the serum with highest HA titre (1:10 240) was the poorest.

It appears that the opsonizing activity of sera does not reflect the immune status of an animal nor is there any correlation between the HA titre of a serum and its opsonizing activity.

Cross immunity between strains

The results presented in Fig. 7 show the degree of resistance obtained in rabbits immunized with four strains of *S. aureus* to challenge with strain 24276. There was little difference between the immunity induced by strains 24276(w), Wood 46 and Smith E, but the mutant strain 68V5 rendered poor protection against its parent strain 24276.

Because strain 24276(w) was the only one suitable for intradermal challenge in rabbits, this species could not be used for reverse experiments. Mice had to be used to test the immunizing activity of a single strain to challenge with a series of heterologous strains.

The results given in Table 2 show the marked effect of the challenge dose on the degree of demonstrable immunity. When immunized mice were challenged with 2×10^8 bacteria the immunity was of a low order, while in the groups challenged with 1×10^8 bacteria, which did not give 100 per cent mortality in the control group, the protection was good. There was very little difference between groups which received two injec-

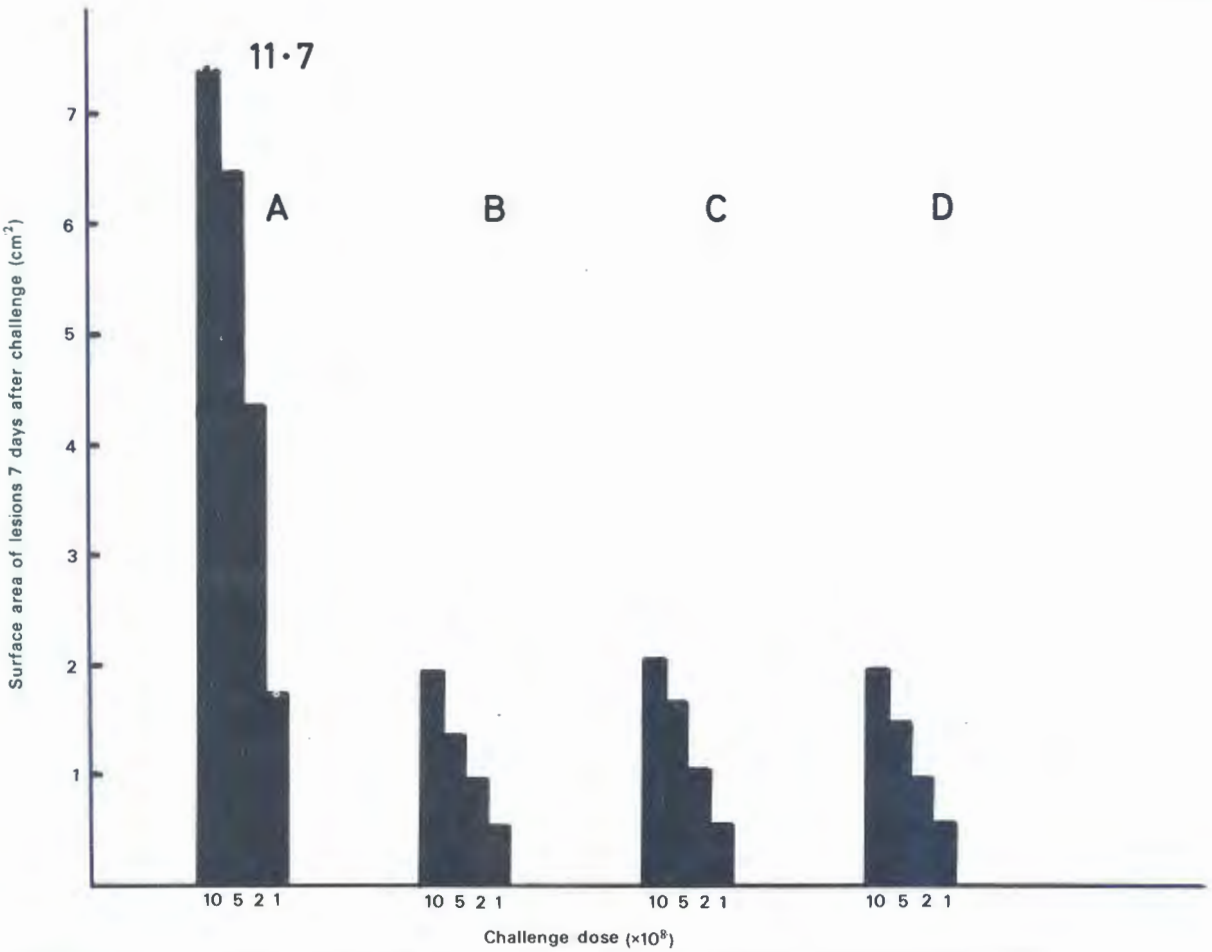

FIG. 3 Comparison of adjuvant cell toxoid, cells only and toxoid only on immunity to skin infections in rabbits.
A = Non-immunized controls; B = Adjuvant cell toxoid; C = Cells only; D = Toxoid only

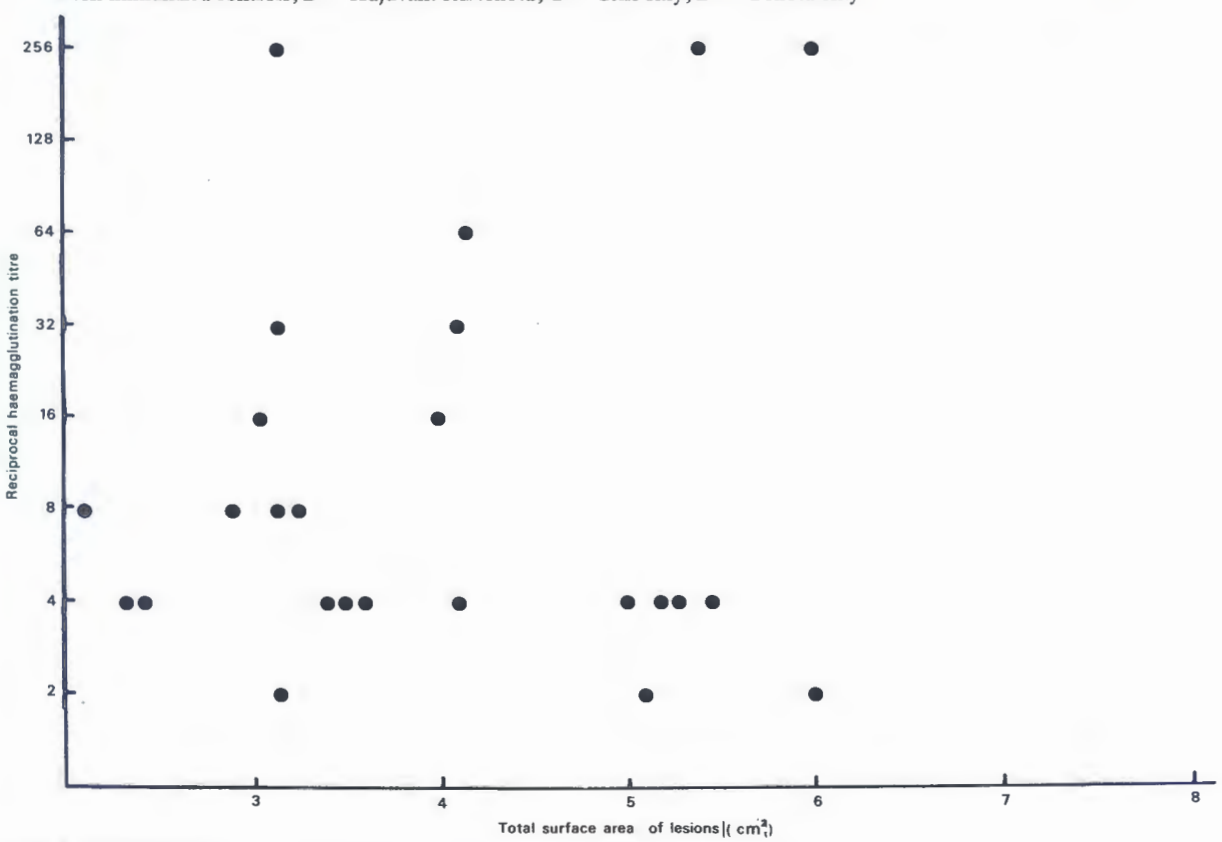

FIG. 4 Relationship of haemagglutination titre and immunity in individual rabbits

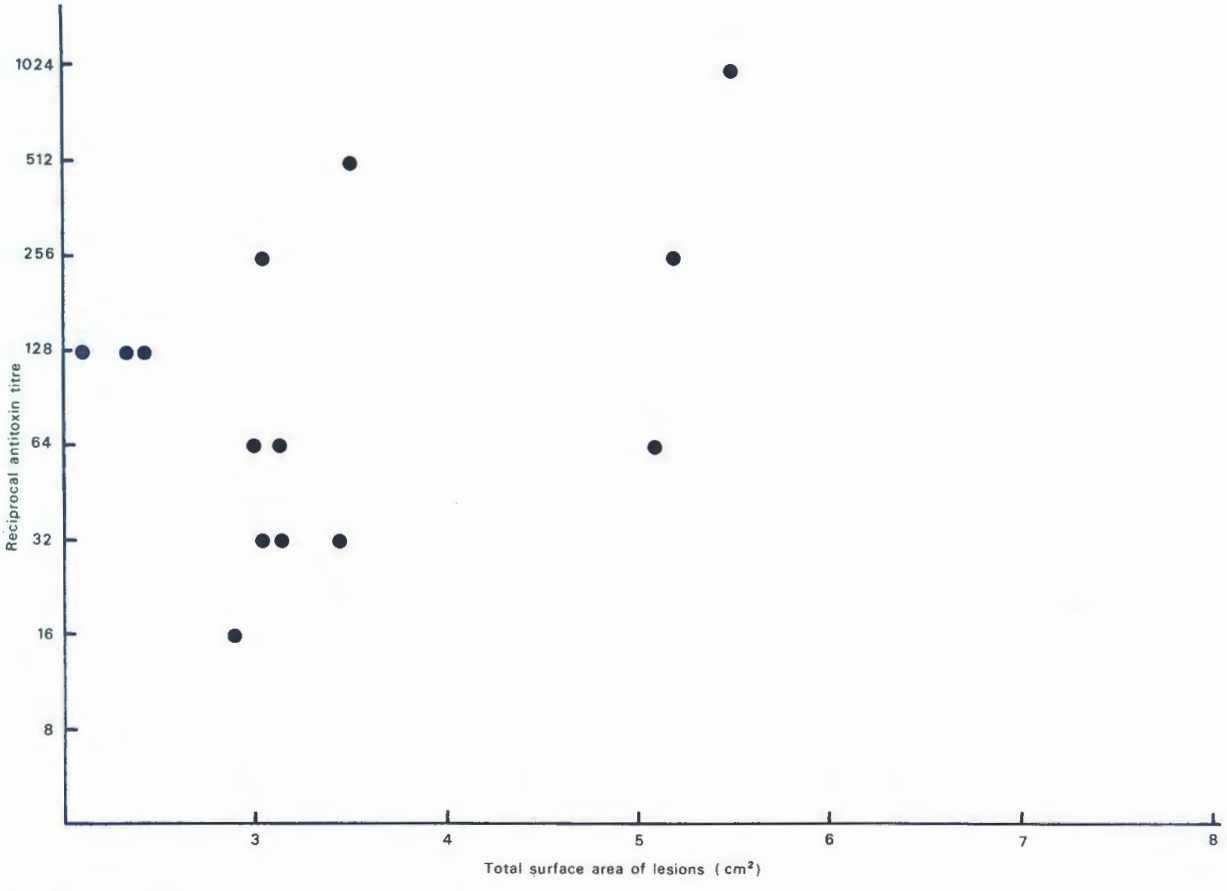

FIG. 5 Relationship of beta antitoxin titres and immunity in individual rabbits

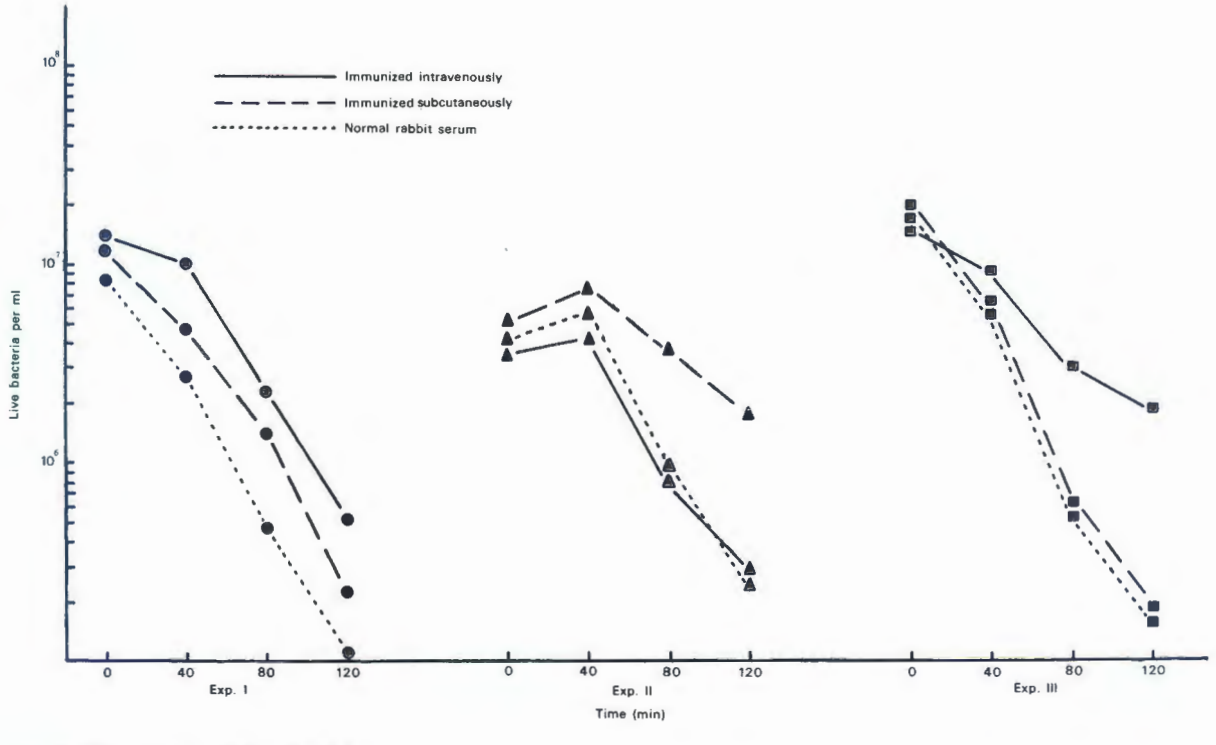

FIG. 6 Opsonizing activity of rabbit sera

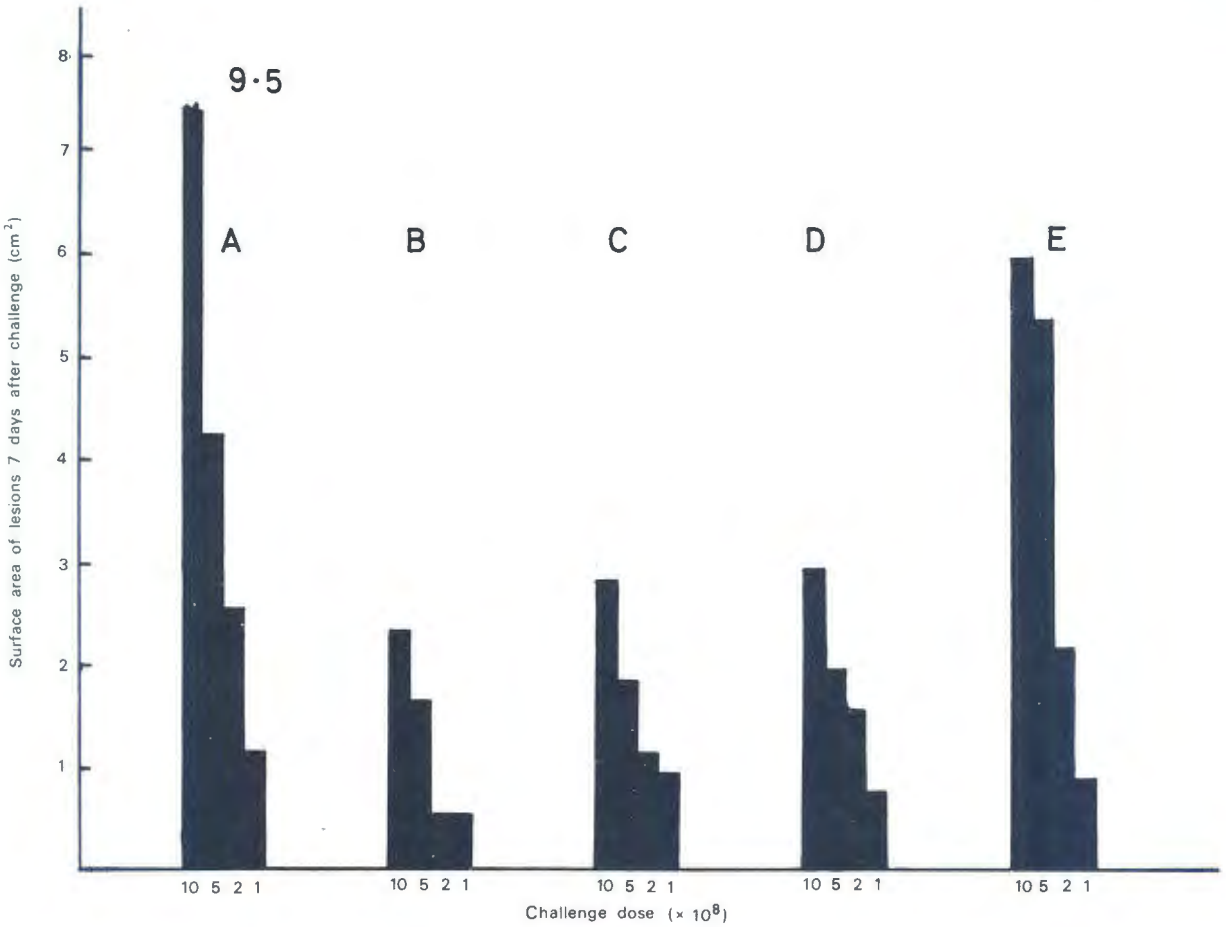

FIG. 7 Immunizing capacity of heterologous strains to challenge with strain 24276(w)
A = Non-immunized controls
B = Immunized with strain 24276
C = „ „ „ Wood 46
D = „ „ „ Smith E
E = „ „ „ 68V5

tions of 0,2 ml vaccine and those that received two injections of 0,1 ml, but two injections of 0,05 ml vaccine gave a poorer immunity.

TABLE 2 *Influence of vaccine dose and challenge level of immunity to* S. aureus *in mice*

Vaccine	Vaccine dose (ml)	Challenge dose (x10⁸)	Deaths/10
Bacteria only	0,2	2	6
„ „	0,2	1	0
„ „	0,2	0,5	0
„ „	0,1	2	6
„ „	0,1	1	1
„ „	0,1	0,5	0
„ „	0,05	2	3
„ „	0,05	1	4
„ „	0,05	0,5	0
Adjuvant cell toxoid	0,2	2	4
„ „ „	0,2	1	1
„ „ „	0,2	0,5	1
„ „ „	0,1	2	6
„ „ „	0,1	1	2
„ „ „	0,1	0,5	1
„ „ „	0,05	2	6
„ „ „	0,05	1	5
„ „ „	0,05	0,5	8
Controls	—	2	10
„	—	1	8
„	—	0,5	6

When the results are viewed as a whole it is clear that vaccine containing bacteria alone gave as good an immunity as bacteria augmented by toxoid. This observation corresponds to the findings in rabbits.

TABLE 3 *Immunity of mice immunized with* S. aureus *strain 24276(w) and challenged with heterologous strains*

Challenge strain	Challenge dose (x10⁸)	Deaths/10	
		Mice immunized with *S. aureus* strain 24276(w)	Non-immunized control mice
24276(w)	2	8	10
„	1	2	10
24276(G)	2	9	10
„	1	10	10
Smith E	2	9	10
„	1	10	10
S38	2	5	10
„	1	5	5
11207	2	2	7
„	1	0	0
Wood 46	2	0	8
„	1	0	0
949	2	9	9
„	1	5	8
Rabec	2	10	9
„	1	9	8
1003	2	9	1
„	1	6	2

In the following experiment all the mice were immunized by administering two subcutaneous injections of 0,1 ml of bacterial vaccine prepared from strain 24276(w). Groups of 10 mice were challenged with 2×10^8 and 1×10^8 bacteria respectively of a number of heterologous strains. The results are shown in Table 3. Strain 24276(w) gave a demonstrable immunity to homologous challenge as well as to strains Wood 46 and 1003, but did not protect against challenge with the other strains.

Discussion

Cohn & Morse (1959) have shown that the phagocytes from immunized rabbits do not differ from normal phagocytes with respect to their ability to engulf and destroy staphylococci and they thus proved that cellular immunity is not involved in staphylococcus immunity. On the other hand, it has been repeatedly shown that serum from immunized animals can passively protect mice (Farrell & Kitching, 1940; Fisher, 1959; Yoshida & Ekstedt, 1968) and Johnson (1966) has demonstrated the beneficial therapeutic effect of gamma globulin in the treatment of staphylococcal infections. It is therefore apparent that resistance to staphylococci is due to a humoral defence mechanism.

The nature of this defence mechanism is however poorly understood and numerous attempts have been made to identify antibodies to a specific toxin or antigen and to correlate the presence of these antibodies with immunity (Cameron, 1963). McLeod, Hall & Frohman (1963) claim that the bactericidal activity of serum from immunized animals is correlated with immunity, but Cybulska & Jeljaszewicz (1966) have shown that this *in vitro* phenomenon has no importance as a defence mechanism against staphylococci.

The results presented in this paper also show that there is no direct relationship between beta antitoxin titres or haemagglutination titres and immunity. Other authors have found some degree of correlation with haemagglutination titres (Yoshida & Ekstedt, 1968) while others have not (Angyal, Laczay & Csapo, 1967). It is generally accepted that there are no valid serological criteria for measuring immunity to staphylococci (Rogers & Melly, 1965).

The results also show that there is no correlation between the opsonizing activity and haemagglutination titre of sera and immunity. These findings agree with the results of Ekstedt (1965), who has shown that even serum from colostrum-deprived piglets will support phagocytosis. It should, however, be remembered that these results pertain to local chronic infections and it may yet be that effective opsonization may play a more prominent role in acute cases where systemic infection is the cause of death.

It seems, therefore, that no single antibody to a specific antigen is responsible, but that the mechanism of immunity to *S. aureus* is a highly complex process involving numerous antigen-antibody reactions which cumulatively lead to effective immunity. The factors concerned are probably as diverse as the antigenic composition of the organism itself. At present the only acceptable method of assaying the value of a vaccine is by active immunization and challenge of experimental animals or alternatively by determining the passive protective potency of the serum of immunized animals. This assay method is closely correlated with immunity (Angyal *et al.*, 1967). Yoshida & Ekstedt (1968) have shown that protection is primarily due to antibodies of the IgM class.

According to Rogers & Melly (1965) the route and physical state in which an antigen is presented to the body are critical factors governing the immune response. With this fact in view different authors have employed a variety of vaccines prepared from physically altered staphylococci cell walls and extracts. Greenberg & Cooper (1960), Greenberg, Cooper & Healy (1961), Greenberg & Le Riche (1961) and Greenberg (1968), have successfully used an enzyme-lyzed polyvalent vaccine in rabbits but Lepper (1967) reported poor results with the same vaccine in goats. Cell wall vaccines have been administered to both goats and mice with variable results (Singleton, Ross, Stedman & Chanter, 1967; Hill, 1969). San Clemente, Renshaw, Fisher & Drury (1966) and San Clemente (1970) found that animals responded differently to certain staphylococcal antigens depending on whether they were administered alone or in combination.

All these data clearly indicate that complex antigens are preferable when overall satisfactory immunity is required. Furthermore, Angyal *et al.* (1967) stress the fact that very carefully controlled and defined production procedures are necessary to ensure success and Ekstedt & Yoshida (1969) have shown that the composition of the medium materially influences the immunological properties of the bacterial cells.

The attempts to elucidate the problem of strain specific immunity were inconclusive. Some degree of cross protection could be demonstrated with certain strains but not with others. The virulence of strains, however, varies so much that in order to obtain reliable results it will be necessary to conduct extensive experiments with graded doses of vaccine and challenge at numerous levels of exposure. This approach has been used successfully for the assay of *Pasteurella* vaccines (Cameron & Smit, 1970).

Summary

By using skin infection in rabbits as the test system, it was shown that these animals may be equally well immunized by either the intravenous or subcutaneous route and that two injections are sufficient. A good immunity could be obtained with a vaccine prepared from bacteria alone, with a whole culture vaccine or with a vaccine containing both bacteria and toxoid.

Immunized animals had higher antitoxin levels and haemagglutination titres than non-immunized controls, but no correlation could be demonstrated between these titres and the degree of immunity. The opsonizing activity of sera also had no relationship to the haemagglutination titres or actual immunity.

Experiments designed to examine the degree of cross immunity between *S. aureus* strains, gave inconclusive results.

Acknowledgements

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