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COMPOSITION AND EVALUATION OF THE EFFICACY OF A *STAPHYLOCOCCUS AUREUS* VACCINE

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ABSTRACT

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An alum-precipitated *Staphylococcus aureus* vaccine, composed of a formalin-inactivated whole culture of a strain which produces Smith surface antigen and combined with the whole culture of a highly toxigenic strain, was found to afford a good immunity to staphylococcal skin infection in rabbits.

Three injections of the vaccine provided immunity which lasted for at least 6 months against a primarily pyogenic strain of *S. aureus* and for at least 3 months against a toxigenic strain.

From experiments using vaccines prepared from cells or toxoid only, it was deduced that, although there is a measure of strain specific immunity, a good heterologous immunity can be established with a combined product provided that it contains adequate quantities of toxoid.

The use of such a vaccine as a potential aid in the control of bovine staphylococcal mastitis is discussed.

Résumé

COMPOSITION D'UN VACCIN DE STAPHYLOCOCCUS AUREUS *ET ÉVALUATION DE SON EFFICACITÉ*

Un vaccin de Staphylococcus aureus *précipité à l'alun, composé d'une culture entière, inactivée au formol, d'une souche qui produit l'antigène de surface Smith, et combiné à une souche hautement toxigène, s'est avéré procurer une bonne immunité à l'infection staphylococcique cutanée chez le lapin.*

Trois injections du vaccin ont suscité une immunité d'une durée de 6 mois au moins contre une souche primairement pyogène de S. aureus. *Contre une souche toxigène l'immunité a persisté au moins 3 mois.*

D'après des expériences utilisant des vaccins préparés à partir de cellules uniquement ou de toxoïdes uniquement, on a conclu que, bien qu'il existe une mesure d'immunité souche-spécifique, une bonne immunité hétérologue peut être établie avec un produit combiné pourvu qu'il contienne des quantités suffisantes de toxoïdes.

On discute la possibilité d'employer un tel vaccin pour aider au contrôle de la mastite bovine à staphylocoques.

INTRODUCTION

In a previous investigation it was found that no single serological test or *in vitro* assay could be directly correlated with actual immunity to *Staphylococcus aureus* skin infection in rabbits. We deduced that immunity is dependent on the sum total of numerous immunological reactions and therefore the only reliable method of assessing the immune status of an animal after immunization is by actual challenge with live bacteria (Cameron, 1971). A prerequisite for effective immunization is therefore the formulation of a suitable vaccine which should contain not only somatic cell antigens, such as cell wall teichoic acid (Cameron, 1969) and particularly Smith surface antigen (Mudd, Yoshida, Li & Lenhart, 1963; Cameron, 1966; Ekstedt, 1966) but also toxoid, since both are involved in establishing immunity (Koenig, Melly & Rogers, 1962).

There is a divergence of opinion regarding the role of alpha haemolysin in the establishment of infection (Foster, 1963; Anderson, 1976) but there is little doubt that alpha antitoxin is essential for protection against toxigenic strains (Derbyshire, 1962).

Since *S. aureus* grows well in numerous media, it is not difficult to obtain dense cultures, but the nutritional and physical requirements for toxin production are more exacting and much work has been done on defining optimum conditions (Cameron, 1965; Smith, Loken & Lindorfer, 1964). A practical compromise for routine vaccine production is thus desirable and we consequently examined some media for their ability to provide both cell growth and toxin production in the absence of CO_2 by using shake cultures (Miyasaki & Takarabe, 1961).

Although a good immunity to challenge with homologous strains has been reported by numerous workers and confirmed by our results (Cameron, 1966 & 1971), there is no clarity regarding the question of strain specific immunity. Weld & Rogers (1960) found that immunized rabbits developed high titre staphylococcal haemagglutinins to homologous strains and that the sera also showed a low level of cross reactions to various heterologous strains. Angyal, Laczay & Csapó (1967) administered autogenous vaccine, Smith diffuse vaccine and Smith compact vaccine to 49 patients suffering from chronic staphylococcal skin diseases. The good results obtained with the Smith diffuse and autogenous vaccines were accompanied by a rise in the phagocytic index and mouse protective antibodies, thus indicating an appreciable degree of cross-immunity. These results are supported by the findings of Yoshida, Ichiman & Ohtomo (1975) who, in addition, found that Smith compact type strains were able to absorb antibodies from antisera prepared with the Smith compact type of strain. Rogers & Melly (1962) likewise showed that the normal uncapsulated strains would produce antibodies which promote the phagocytosis of Smith type of strains.

Stamp (1964) and Stamp & Edwards (1964) claimed that immunity to *S. aureus* was not strain specific. In support of this contention, Hill (1969) showed that a deoxycholate extract residue from a particular strain protected mice against a range of heterologous

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strains of *S. aureus*. Angyal (1966) reached virtually identical conclusions. Conversely, Greenberg & Cooper (1960) maintained that it was necessary to include a variety of strains in a vaccine in order to obtain a wide spectrum of immunity. Taking into account such divergence of opinion, we deemed it necessary to investigate this aspect further.

Much of the work that has been done on *S. aureus* has revolved round its mechanisms of virulence and an assessment of the antigenicity and immunogenicity of toxoids and somatic antigens, but little has been done regarding optimum immunization schedules and the duration of immunity. Because of the scant information on these aspects of *S. aureus*, we included both in our studies.

Materials and Methods

Experimental animals

Groups of six 4–6-month-old New Zealand-white type albino rabbits were used to assay the immunogenicity of the various vaccine preparations. The number of groups varied from one experiment to another. They were housed in wire cages and fed a pelleted balanced ration.

Culture media

The following media were used:

(i) Medium 110 (Oxoid*) was slightly modified in that the NaCl content was reduced to 0,5% and mannitol was replaced with glucose; (ii) Tryptone soya broth (Oxoid*) (TSB); (iii) Pattison and Matthews' broth (P & M) (Pattison & Matthews, 1957); (iv) Leonard and Holm's medium (L & H) (Leonard & Holm, 1935) from which the agar was omitted; (v) Bernheimer & Schwartz's medium (B & S) (Bernheimer & Schwartz, 1963) modified by replacing the yeast diffusate with 1,0% yeast extract (Oxoid*); (vi) Onderstepoort nutrient broth (OPB) (Cameron, 1965); (vii) Brain Heart Infusion broth (BHI) (Difco); and (viii) Wiley's glycerol broth (W) (Wiley, 1961).

All the media were prepared according to the published instructions, distributed in 200 mℓ and 500 mℓ quantities in Roux flasks and 2 ℓ Pivitsky flasks, respectively, and sterilized at 120 °C for either 30 min or 60 min.

Bacterial strains

S. aureus (Wood 46) was obtained from Dr R. K. Lindorfer**. Strain 24276 (68 V5) W is a Smith compact type of organism and has been described in detail (Cameron, 1966 & 1969). Strain S38 (4) as well as all the other strains used in this study was isolated from cases of bovine mastitis. Strain 24276 (68 V5) W gives rise to primarily purulent lesions while Strain S38 (4) gives essentially necrotic lessions.

Assay of growth and toxin production

Six Roux flasks of each of the media to be assayed were inoculated with 2,0 mℓ of a serum broth culture of the strains used. The flasks were then incubated for 18 h at 37 °C in a horizontal shaker. The packed cell volume (pcv) was measured by means of Hopkins' tubes.

Assays for toxin production were based on the method described by Cruickshank, Duguid, Marmion & Swain (1975). Alpha haemolysin was titrated by making twofold serial dilutions of the culture supernatant fluid in 1,0 mℓ volumes in 0,15 M phosphate buffered saline (pH 6,8) to which 0,1% gelatin was added. To each tube 0,1 mℓ of a 10% suspension of washed rabbit erythrocytes was added and the tubes were then incubated at 37 °C for 60 min. The endpoint was taken as the reciprocal of the highest dilution showing complete haemolysis. Beta haemolysin was titrated in a similar way except that the buffer used contained 0,02% $MgSO_4.7H_2O$, but no gelatin, and had a pH of 7,3. Sheep erythrocytes were used instead of rabbit erythrocytes and incubation at 37 °C for 60 min was followed by keeping the tubes at 4 °C for 60 min.

Preparation of vaccines

The strains which were used for the various preparations were grown in 500 mℓ volumes of P & M broth as outlined above. Inactivation of the bacteria and toxoiding was accomplished by the addition of of 1,0% formalin and keeping the flasks at 37 °C for 10 days. When only toxoid was required, the bacteria were first removed by centrifugation.

When required, the inactivated culture was precipitated by the addition of 10 mℓ of an 11% solution of potassium alum to 100 mℓ of culture or toxoid.

Oil adjuvant vaccines were prepared as described by Cameron & Fuls (1978). These include 'Bluetongue adjuvant' (BT) and modified Burroughs Wellcome adjuvant (BW).

Immunization and challenge of rabbits

In the initial experiments the rabbits were given 2 subcutaneous injections of vaccine with an interval of 4 weeks between the injections and challenged 10 days after the 2nd injection. For the last 2 experiments, the schedule was adapted to conform with the requirements. In all cases the dosage was 2,0 mℓ.

For challenge purposes, the strains were grown for 18 h at 37 °C in shake cultures. The cells were collected by centrifugation and resuspended in saline to a density of 2,0% and ½ and ⅕ dilutions prepared. The rabbits were shaved on the day before challenge and 0,1 mℓ of each dilution of 2 challenge strains was injected into both flanks of each rabbit, care being taken to avoid areas of active hair growth. In 1 experiment only undiluted and ½ dilution of the challenge material was used.

After 7 days the diameter of the lesions was measured and the surface area calculated. The histograms show the average surface area of the lesions for each dilution.

Results

Vaccine production

From the results of a preliminary experiment shown in Table 1, it is apparent that BHI broth is the medium of choice with respect to both toxin production and cell yield. It is too expensive for mass production, however, and further experiments were done using TSB, P & M and OPB medium only.

A further examination showed that P & M broth consistently yielded both high titres of alpha and beta haemolysin and a dense concentration of cells (Table 2) and was consequently selected for routine vaccine production.

* The Oxoid Manual, 2nd Ed., p 197

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TABLE 1 Comparison of media for toxin production and yield of bacteria in Roux flasks containing 200 mℓ of media

Medium	Alpha haemolysin Wood 46	Beta haemolysin S38 (4)	Cell yield % pcv 24276 (68 V5) W
110	16	16	0,6
TSB	128	512	0,9
P & M	1 024	256	0,9
L & H	640	256	0,5
B & S	64	512	0,3
OPB	544	512	0,8
BHI	3 072	1 024	1,2
W	0	2	0,1

TABLE 2 Examples of toxin titres and cell yield obtained in 500 mℓ shake cultures with Strains S38 (4) and 24276 (68 V5) W in different media

Medium	Strain S38 (4)			Strain 24276 (68 V5) W		
	Alpha haemolysin	Beta haemolysin	Cell yield % pcv	Alpha haemolysin	Beta haemolysin	Cell yield % pcv
TSB	64	256	1,1	2	512	1,0
	128	512	1,0	2	128	1,0
P & M	512	4 096	1,8	64	2 048	1,8
	512	1 024	1,9	16	1 024	1,7
	1 024	256	1,8	64	4 096	2,0
	1 024	2 048	1,8	32	4 096	1,8
OPB	64	256	1,0	8	256	1,3

Effect of adjuvants on immunity

As can be seen from the results shown in Fig. 1, there is no essential difference between the degree of protection afforded by a vaccine without adjuvant and that afforded by either an alum-precipitated vaccine or oil adjuvant vaccines.

Similar results were obtained when immunized rabbits were also challenged with the toxigenic Strain S38 (4) (Fig. 2).

Strain specificity of immunity

The results of an experiment in which immunized rabbits were challenged with 8 different strains of *S. aureus* are shown in Fig. 3.

Apart from Strains 24276 (68 V5) W and S38 (4), none of the other strains was able to establish progressive lesions and the degree of protection against them could thus not be determined. Further experimentation was therefore directed at examining the immunological relationship between the 2 pathogenic strains.

The outcome of this experiment is graphically shown in Fig. 4. Rabbits which were immunized with whole culture vaccine prepared from Strain 24276 (68 V5) W were well protected against challenge with the homologous strain, but they were not particularly resistant to challenge with the more toxigenic Strain S38 (4). Conversely, rabbits immunized with whole culture vaccine of Strain S38 (4) were well protected against both the homologous and the heterologous strains.

The immunizing role of toxoid

The apparent role of toxoid was further demonstrated in an experiment in which groups of rabbits were immunized either with cells only, toxoid only or toxoid plus cells of the respective strains and challenged with both strains. Fig. 5 indicates that cells alone were only able to effect an immunity to challenge with Strain 24276 (68 V5) W. Crude toxoid prepared from Strain 24276 (68 V5) W (which produces only a little alpha toxin but which would contain SSA) protected aginst challenge with the homologous strain but not against Strain S38 (4). On the other hand, Strain S38 (4) toxoid did not give effective protection against Strain 24276 (68 V5) W but did give protection against itself.

Whole culture vaccine of Strain 24276 (68 V5) W gave homologous protection only, whereas the whole culture vaccine of Strain S38 (4) gave both homologous and heterologous protection. Thus it is evident that, although the bacteria alone do not give good cross protection (indicating that they are immunologically different), good cross immunity is obtained with a vaccine containing both cells and toxoid.

Immunization schedules and duration of immunity

All 3 schedules of immunization using a combined vaccine containing both cells and toxoid gave a good immunity, but from the results shown in Fig. 6 it appears that the more intensive schedule comprising 3 injections at 10-day intervals was possibly marginally better than the other 2.

The results shown in Fig. 7 indicate that rabbits given 3 injections of combined alum-precipitated cell toxoid were immune for 3 months to challenge with both strains. At 6 months post-immunization, they were still immune to Strain 24276 (68 V5) W but had lost much of their immunity to Strain S38 (4). At 9 months the non-immunized controls had developed a marked degree of natural resistance and did not develop extensive lesions after infection. The apparent immunity in the immunized group is therefore clouded by this observation and it is consequently impossible to establish what the true role of acquired immunity in protection is at this stage.

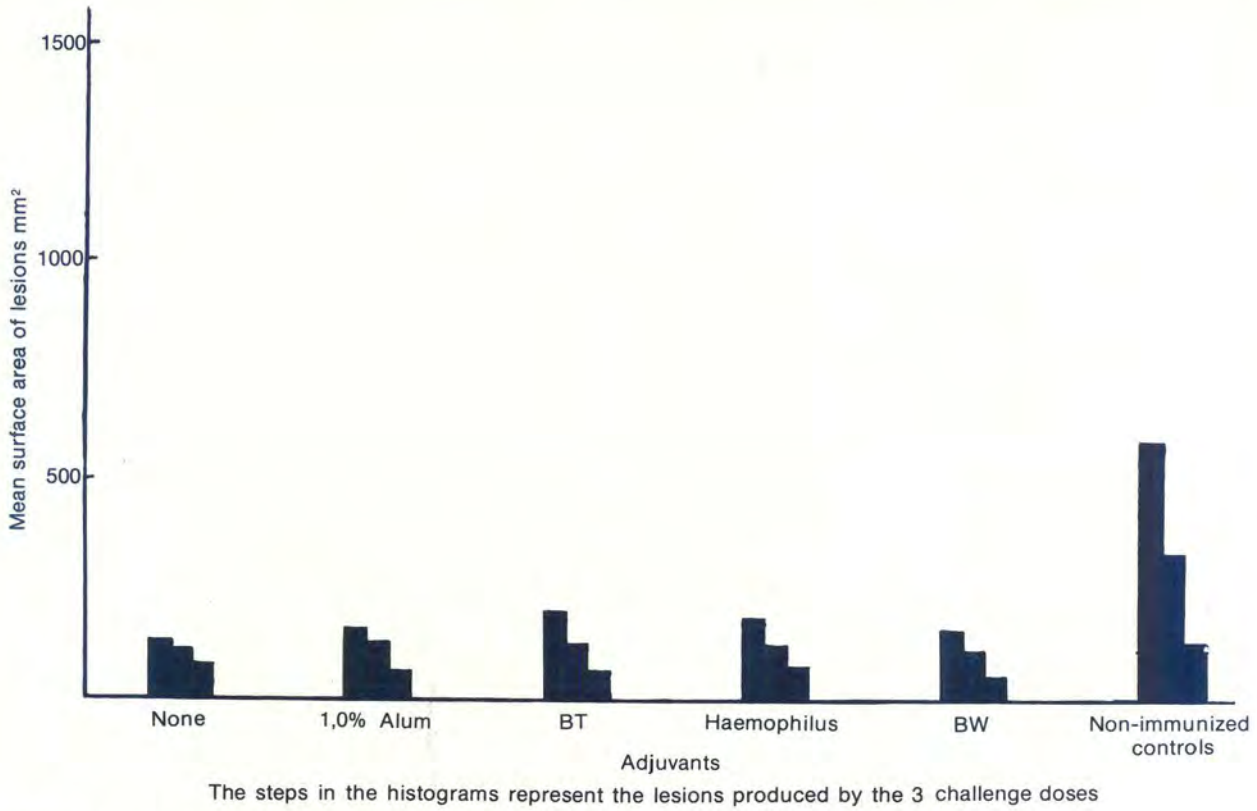

FIG. 1 Effect of adjuvants on immunity to Strain 24276 (68 V5) W

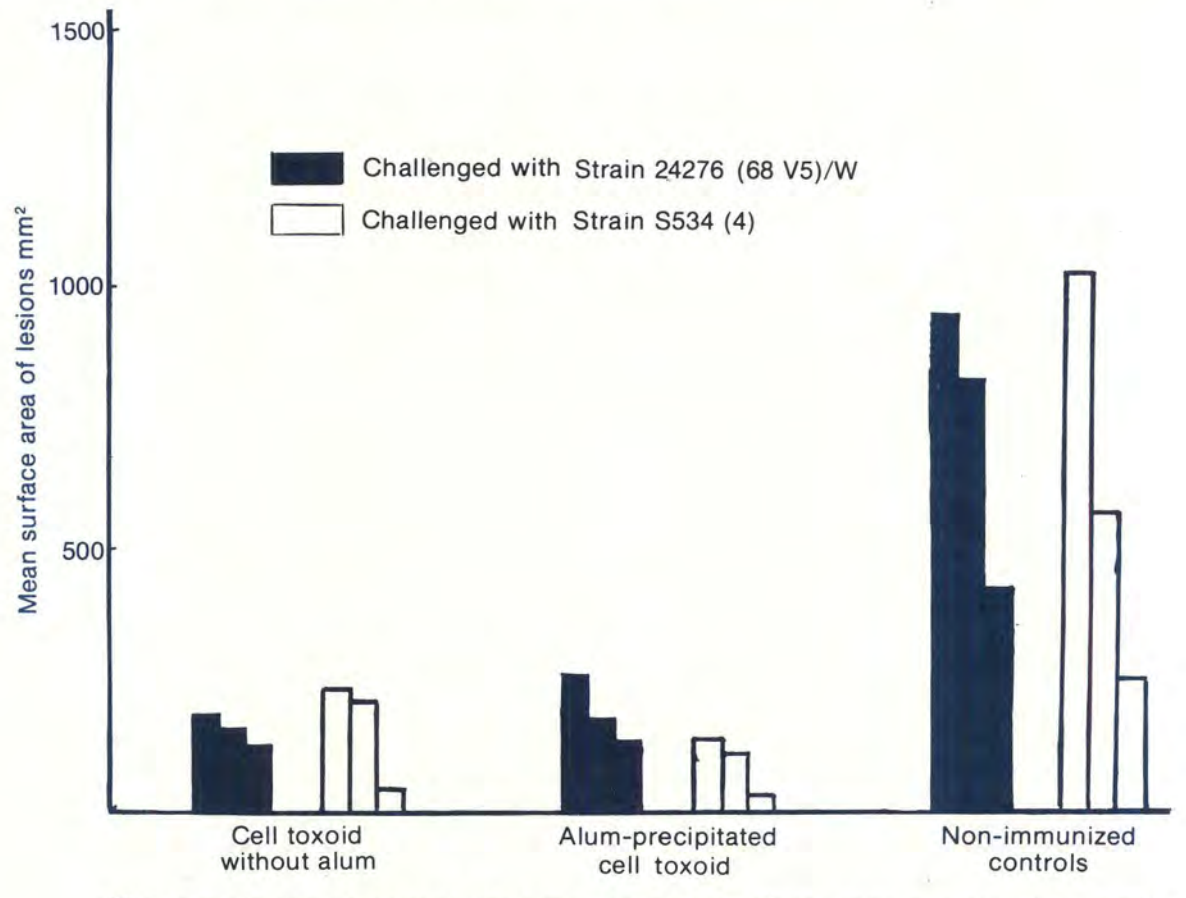

FIG. 2 Comparison of cell toxoid without alum and vaccine precipitated with alum

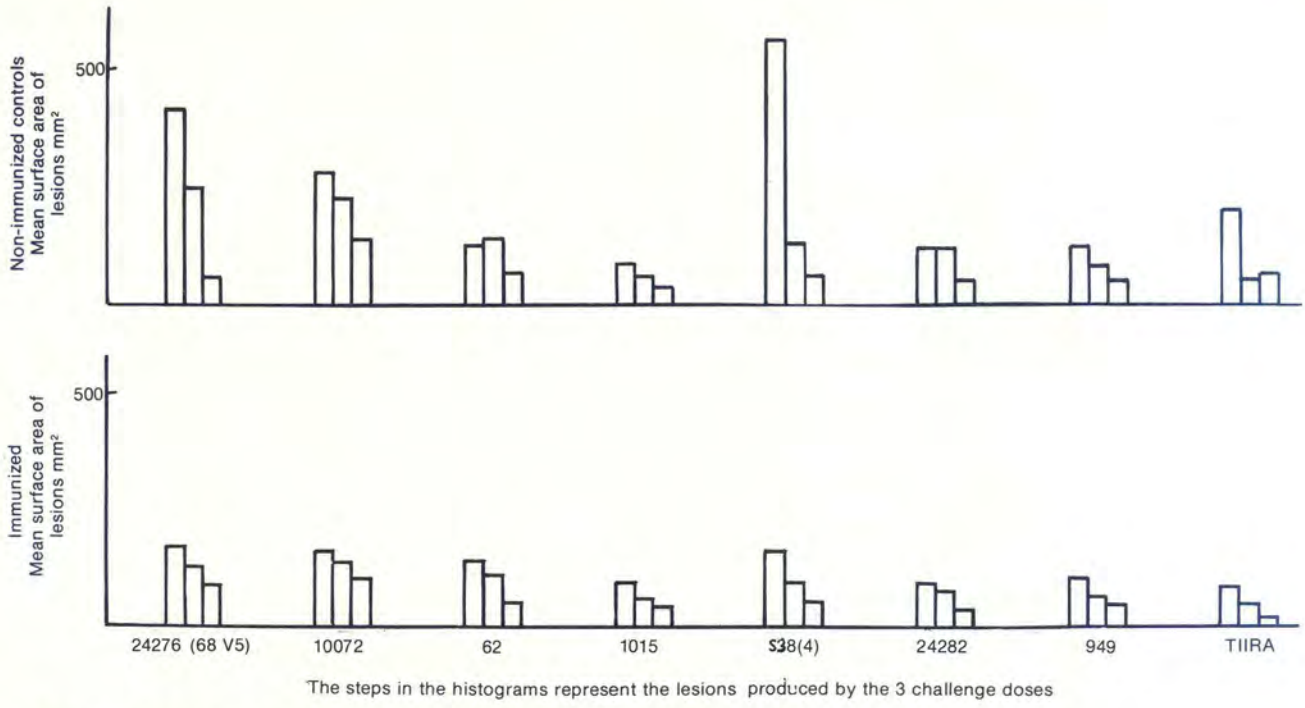

FIG. 3 Immunity afforded by combined adjuvant cell toxoid to heterologous strains

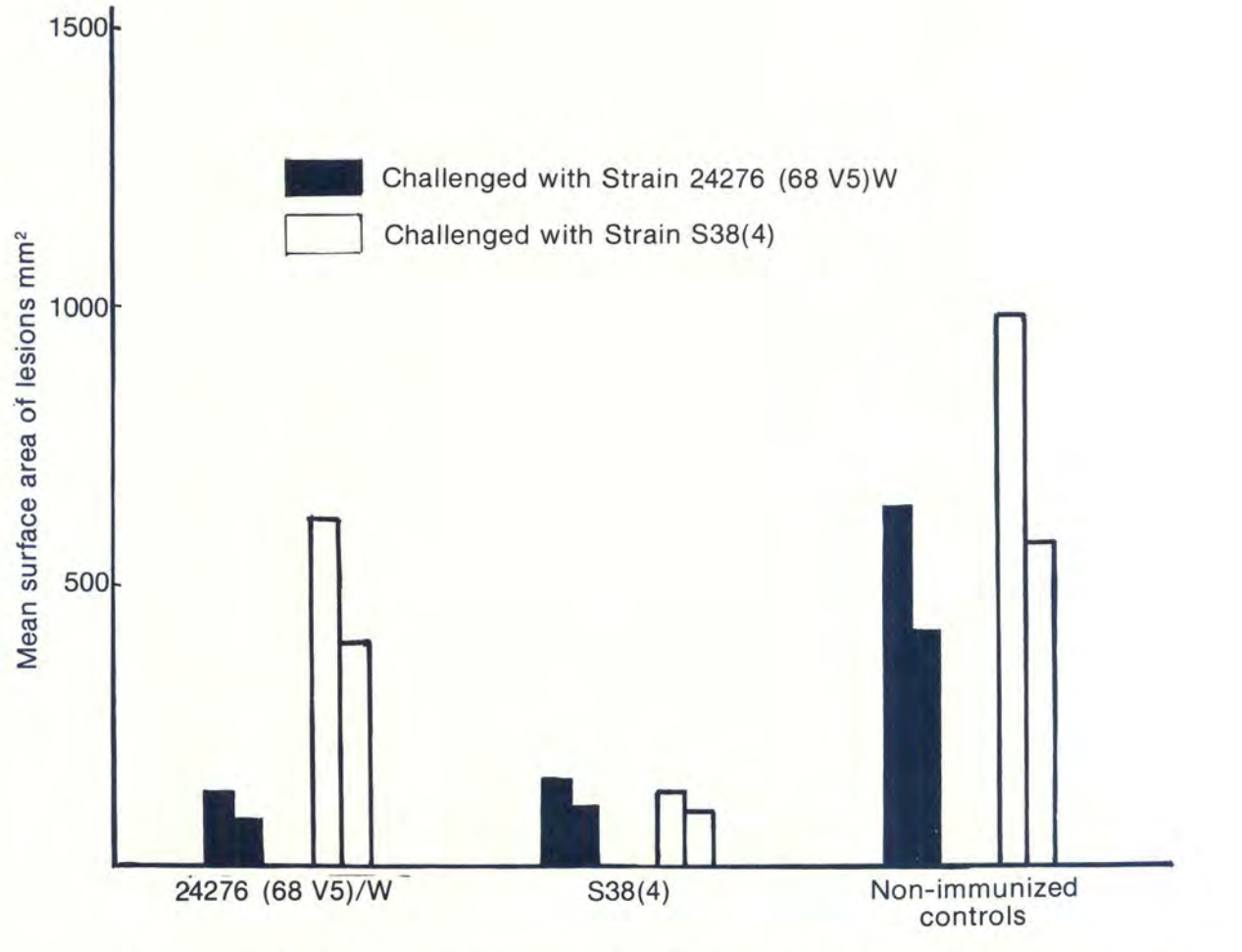

FIG. 4 Immunological relationship between Strains 24276 (68 V5) W and S38 (4)

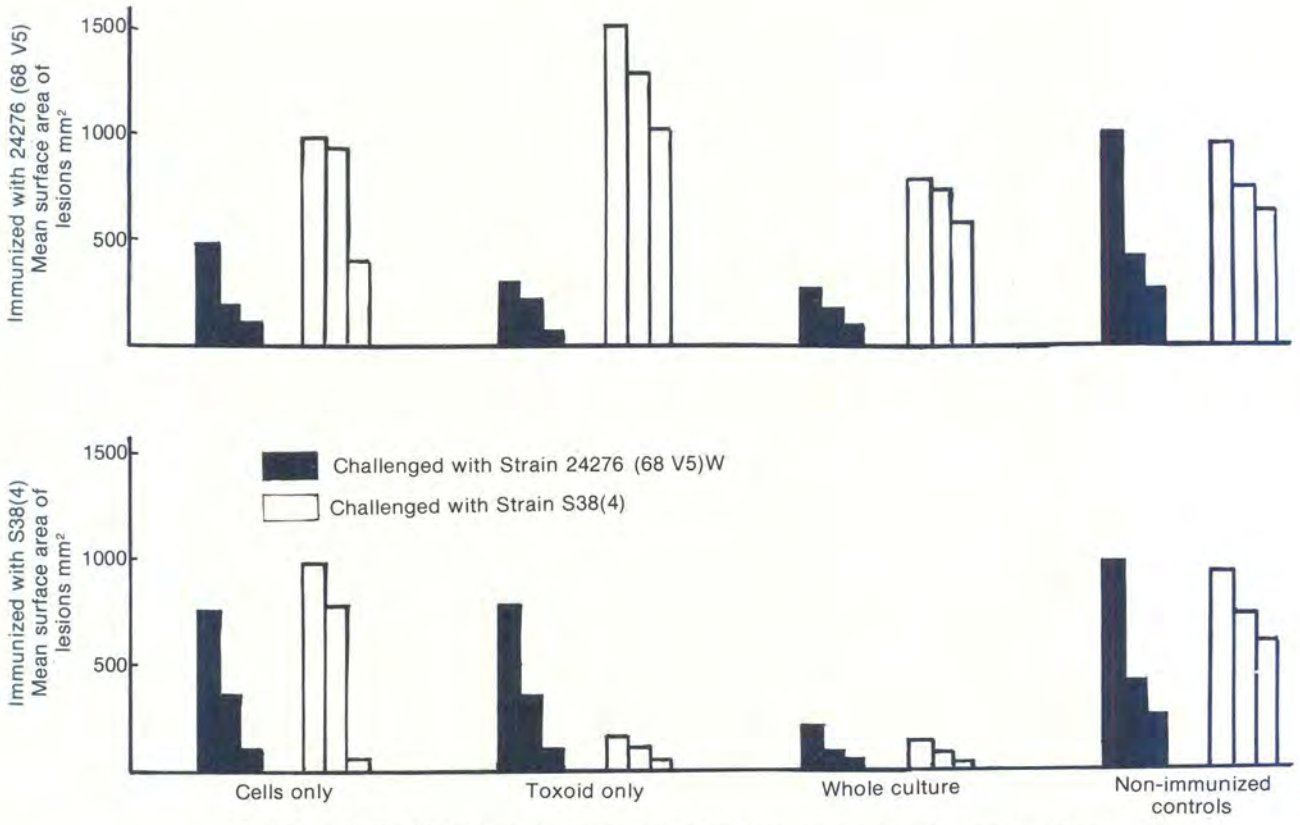

FIG. 5 Comparison of immunizing properties of cells, toxoid and whole culture

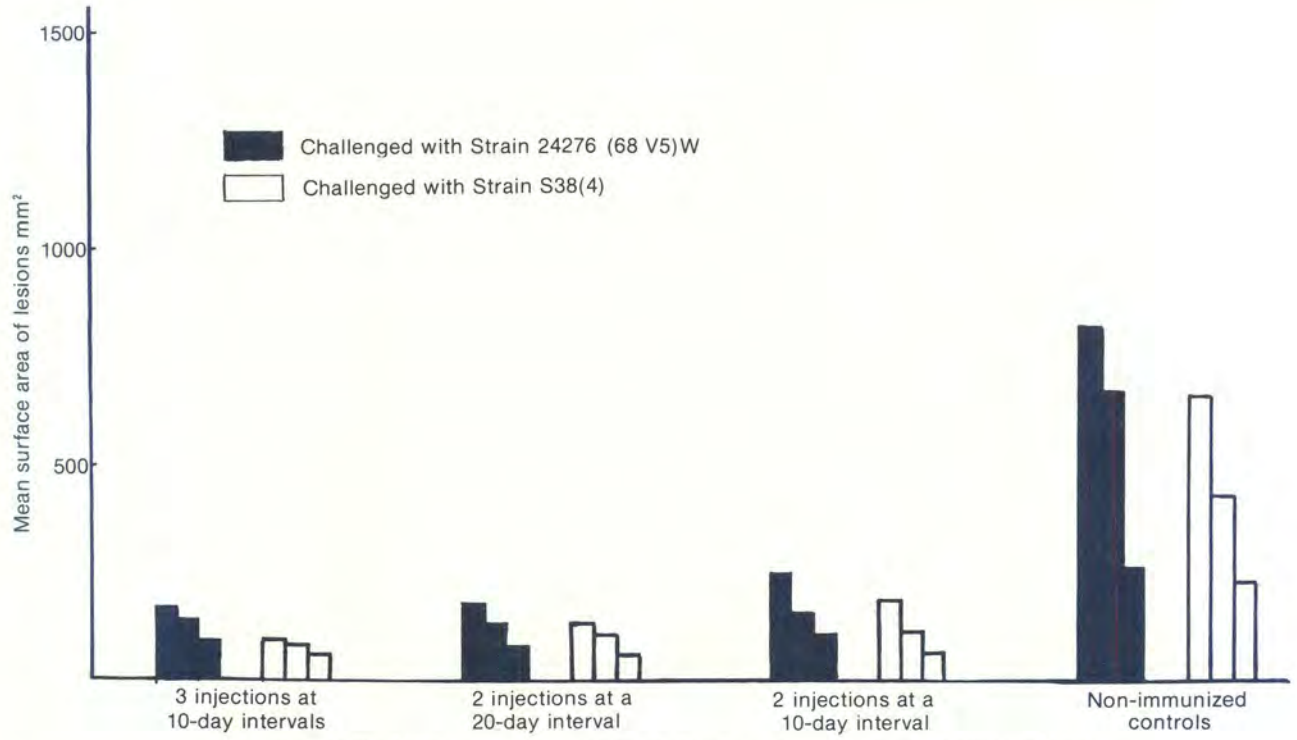

FIG. 6 Comparison of immunization schedules

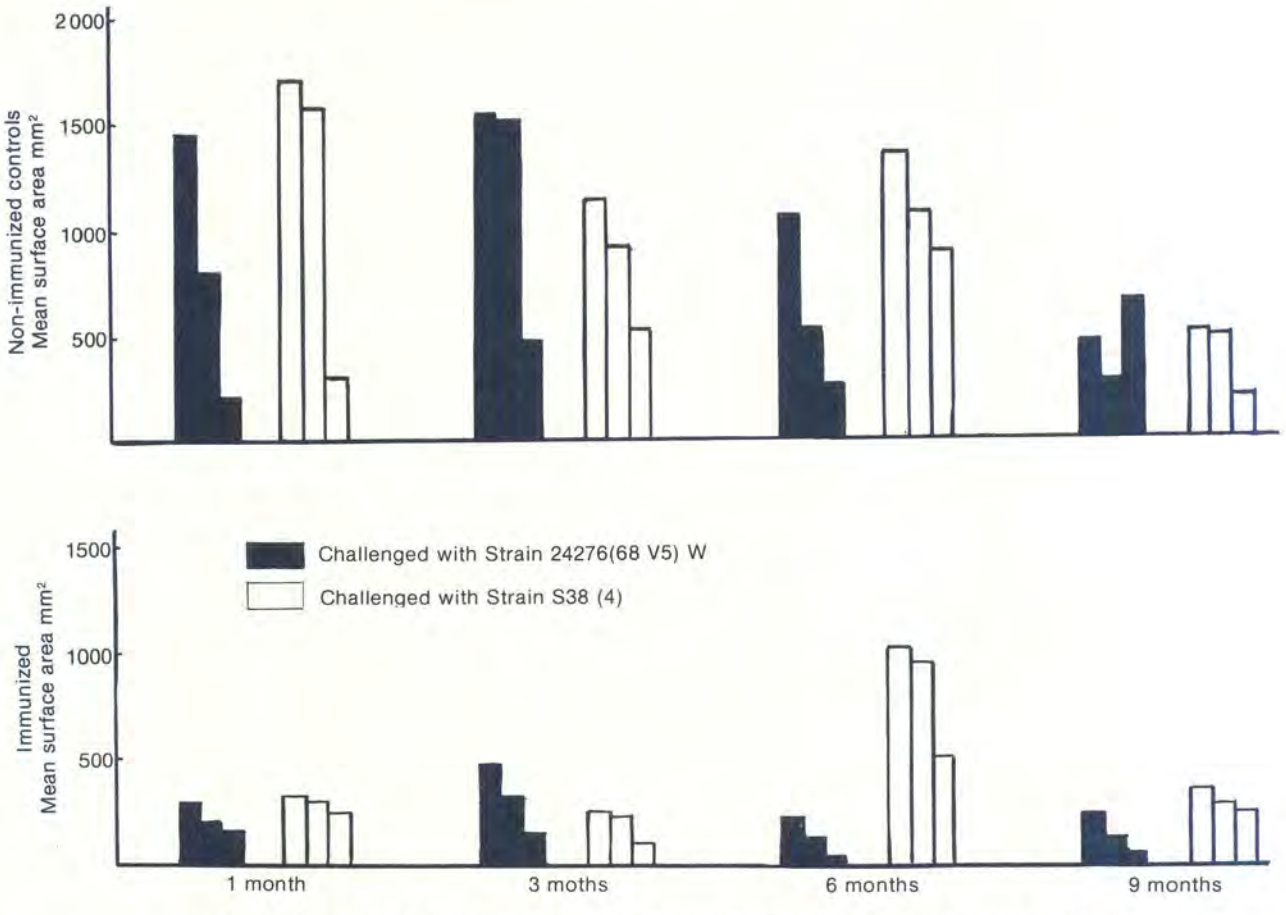

FIG. 7 Duration of immunity in rabbits given 3 injections of combined alum-precipitated cell toxoid at 10-day intervals

Discussion

The results presented in this paper confirm previous findings (Cameron, 1966) that young rabbits can be effectively immunized against staphylococcal skin infections. The duration of immunity is limited, however, and decreases after 4 months, but by this time non-immunized animals have acquired an appreciable degree of natural resistance.

Furthermore, a vaccine containing no toxoid was found to be ineffective in protecting rabbits against infection with a highly toxigenic strain, but a cell toxoid afforded protection against a heterologous strain, a conclusion which agrees with the findings of Downie (1937). There was, however, not reciprocal immunity between the two strains studied when vaccines containing cells only were used, and because of this difference it would be wise to include both strains in a composite vaccine. It can therefore be deduced that, provided a vaccine contains adequate quantities of somatic antigens and toxoid, it would have a wide application and should give protection to an appreciable spectrum of *S. aureus* strains encountered in nature.

The product we have formulated complies with the above requirements and might well be suitable for the immunization of cattle, sheep and goats against staphylococcal mastitis, since it would not only induce more efficient phagocytosis and prevent the multiplication of the organisms, but it would also contribute to the neutralization of toxin which, according to Anderson (1976), are the prime factors involved in the pathogenesis of staphylococcal mastitis. The role of immunization in mastitis control is by no means clear and, according to Norcross & Stark (1969), the major obstacle is the lack of a suitable antigen preparation. The combined vaccine described here may be the answer but its efficacy will have to be tested under field conditions.

The feasibility of effective parenteral immunization is supported not only by the work of Mukkur & Tewari (1975), who demonstrated the presence of antistaphylococcal antibodies in the colostrum of immunized cows, but also by the findings of Watson & Lascelles (1975), although both groups of workers used oil adjuvant vaccines.

Apart from conventional parenteral immunization a second avenue which should be investigated is the use of the intra-mammary route as advocated by Lascelles, MacKenzie & Outteridge (1971). This approach has much promise but is also fraught with difficulties. Not only do staphylococcal antigens induce a leucocytosis when injected into the udder but they may also induce a state of hypersensitivity (Kowalski & Berman, 1971; Targowski & Berman, 1975). This situation may, however, be beneficial since it has been found that the inflammatory response elicited by a hypersensitivity reaction could contribute to non-specific immunity (Florman, 1968; Taubler, Grieb & Mudd, 1970; Easmon & Glynn, 1975).

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