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The Role of Humoral Immunity and Acute Inflammation in Protection against Staphyloccocal Dermonecrosis

C. S. F. EASMON AND A. A. GLYNN

Bacteriology Department, Wright-Fleming Institute, St Mary's Hospital Medical School, London

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Summary. Mice were protected against the dermonecrotic effects of *Staphylococcus aureus* by previous infection with either coagulase-positive or coagulase-negative strains or by immunization with α-toxin. Passive protection was conferred by serum from previously infected mice or by α-antitoxin.

While only some of these methods were associated with circulating α-antitoxin, in all cases there was a brisk early inflammatory response to infection. Furthermore, if the capacity of well immunized mice to mount such a response was removed, they were no longer protected against dermonecrosis. Conversely, non-immune mice developed little or no necrosis if the staphylococci were injected into areas of pre-existing non-specific acute inflammation whether these had been produced chemically or immunologically.

It is suggested that in this model of local infection with *S. aureus* an early inflammatory response, however provoked, is the major protective factor. Though specific neutralizing actions of antibodies are not excluded, the most important result of antibody–antigen reaction is to cause local inflammation by some form of immediate hypersensitivity.

INTRODUCTION

Of the many potential virulence factors produced by *Staphylococcus aureus* α-toxin has received most attention. Nevertheless, opinion and evidence is divided as to its pathogenic significance and the protective value of α-antitoxin. Much of the difficulty is due to the wide variety of experimental models used; for example α-toxin does not kill human leucocytes and dermonecrosis is not a particular feature of staphylococcal infection in man. In the model described by Noble (1965), in which mice are injected subcutaneously with staphylococci mixed with cotton dust, dermonecrosis is often striking. Since this model is much used in our laboratory for experiments on immunity to staphylococci, we wished to define the role of α-toxin in it so as to be able to concentrate on other factors, in particular that found by Hill (1968) in the residue of staphylococcal cell walls extracted with desoxycholate. This material (DOCR) appears to be the factor described by Agarwal (1967b) in virulent but not in avirulent strains of *S. aureus*. It acts as an impedin, suppressing the early inflammatory response to infection and thus allowing the bacteria to grow up more rapidly. The protective value of early inflammation in local infections had previously been pointed out by Miles, Miles and Burke (1957). In mice who have had previous local

Correspondence: Professor A. A. Glynn, St Mary's Hospital Medical School, London W2 1PG.

infections or who have been immunized with DOCR (Hill, 1969), dermonecrosis does not occur and the final lesions are less severe. By contrast, the early phase of inflammation is marked, and cannot be suppressed by virulent strains of *S. aureus* or even by the injection of additional DOCR. We have previously suggested (Easmon, Hamilton and Glynn, 1973) that this may be either a simple quantitative phenomenon, or may occur because in immune, as distinct from normal mice, inflammatory mechanisms which are less easily inhibited by DOCR come into play.

If a major protective event in local staphylococcal infection is the early development of inflammation, then antibodies to any staphylococcal antigen, toxic or not, could protect by provoking an Arthus reaction. Indeed, in situations where acute inflammation is protective, local immediate hypersensitivity can be regarded as the humoral equivalent of the specific production of non-specific active macrophages seen in cell-mediated immunity. There is, of course, also room for more specific protective mechanisms.

We report here some experiments to test this hypothesis. The production of dermonecrosis and local lesions following the injection of *S. aureus* on cotton dust was examined in normal mice and in mice immunized with strains of virulent and non-virulent *S. aureus*, coagulase-negative staphylococci, micrococci, or α-toxin. The effect of passive transfer of immune serum from previously infected mice or of antibody to α-toxin was also observed. In other experiments the inflammatory response to infection was suppressed by prior administration of anti-inflammatory agents or exacerbated by the prior but coincident injection of glycogen.

Finally, staphylococci mixed with a neutral antigen bovine serum albumin (BSA) were injected into mice passively sensitized with anti-BSA. In other words the organisms were injected at the site of a passively produced Arthus reaction.

MATERIALS AND METHODS

Mice

Female outbred Wright–Fleming Institute strain mice, weighing 25–30 grams were used.

Bacteria

The virulent strains of *S. aureus*, PS80 (NCTC 9789), Fisher and Orbach, the relatively avirulent *S. aureus* Wood 46 (NCTC 7121) and the coagulase-negative strains M278, and *Micrococcus hyicus* (NCTC 10350) have been described by Agarwal (1967b). The coagulase-negative strains 1042 and 1061 were from the Wright–Fleming culture collection.

Strains were grown in 0·1 per cent glucose broth. By adjusting the optical density a saline dilution of the culture was made up so that 5 μl of suspension contained $1–3 \times 10^5$ colony forming units (c.f.u.). This volume was absorbed onto a cotton dust plug in a wide bore needle and then injected subcutaneously into the backs of mice depilated with 'Nair'.

Lesion scoring

The lesions were examined at 24 hours and the degree of necrosis expressed as the necrotic index (NI) where

$$\mathrm{NI} = \frac{\Sigma(Dd)}{n}$$

D and d are the major and minor axes of the necrotic lesion measured in mm, n is the

number of mice in the group. This method of scoring, therefore, only takes account of necrosis unlike that used by Noble (1965) and Agarwal (1967a) who scored erythema and pus formation as well.

Immunization

Live staphylococci were given subcutaneously on cotton dust in two doses of 10^3 and 10^5 c.f.u. 1 week apart. The mice were used 2 weeks after the second dose.

For toxin the same time schedule was used but with 0·1 international units of Wellcome α-haemolysin (Wellcome Reagents Ltd).

For passive immunization mice were injected intravenously immediately before infection, with either 4 units of α-antitoxin in a volume of 0·2 ml or 0·2 ml of pooled serum from actively immunized mice.

Anti-inflammatory agents

Anti-thymocyte serum (ATS) was prepared in New Zealand white rabbits according to Levey and Medawar (1966). It was heated at 56° for 30 minutes, filtered through a millipore membrane (0·45 μm) and stored at −20°. The serum was used unabsorbed in a dose of 0·2 ml intraperitoneally which was enough to cause a 90 per cent depletion of circulating lymphocytes in 4 hours. Alternatively predisone (Codelrol, Merck Sharp & Dohme) was given intraperitoneally (i.p.) at a dose of 100 μg/g body weight half an hour before challenge.

Zymosan (Koch Light) was suspended in physiological saline at a concentration of 10 mg/ml and boiled for 1 hour. It was then washed three times in veronal-buffered saline and resuspended in the buffer at the same concentration. Mice received 5 mg i.p. 1 hour before challenge with staphylococci. This was subsequently reduced to 2 mg with no alteration in effect.

Non-specific inflammation

This was induced by the subcutaneous injection of 0·1 ml of 0·1 per cent glycogen. The challenge organisms were injected into the same site 2 hours later.

Production of α-toxin

Bacterial strains were grown for 72 hours in nutrient broth with 0·1 per cent glucose in an atmosphere of 5 per cent CO_2. Dilutions of the culture supernatants (0·5 ml) were tested against sheep red cells (1 ml of 1 per cent) with and without the addition of anti-staphylococcal α-toxin (0·5 ml, 1 u/ml) (Wellcome Reagents Ltd) or against rabbit cells by the method of McClatchy and Rosenblum (1966).

Production of anti-α-toxin

Mice were given two subcutaneous injections of the appropriate organism at an interval of 1 week. Two weeks later they were bled and the serum tested for anti-α-toxin activity by titration in the standard way against staphylococcal α-toxin (Wellcome).

Production of a passive reversed Arthus reaction

Fifty microlitres of anti-bovine serum albumin was given subcutaneously, followed 1 hour or later by 1 mg BSA. Controls were given antibody alone or normal rabbit serum alone or followed by BSA.

RESULTS

IMMUNIZATION OF MICE WITH STRAINS OF *Staphylococcus aureus*

Mice given two injections of live *S. aureus* of the virulent strains PS80 and Fisher or of the non-virulent strain Wood 46 were well protected against dermonecrosis when subsequently challenged with 10^5 c.f.u. on cotton dust of any of the three strains in Table 1.

TABLE 1

EFFECT OF IMMUNIZATION BY *S. aureus* (10^5 *c.f.u.*) ON *S. aureus* LESIONS IN MICE

Challenge strain (10^5 c.f.u.)*	Immunizing strain (10^5 c.f.u.)*†							
	0		PS80		Fisher		Wood 46	
	A	B	A	B	A	B	A	B
PS80	11	32	0	0	0	0	1	0·3
Fisher	12	54	0	0	0	0	1	0·5
Wood 46	4	7·5	0	0	0	0	0	0

* Virulent strains PS80 and Fisher; non-virulent strain Wood 46.
† A = number of mice with necrosis in a group of twelve; B = mean necrotic index for the group.

Immunizing doses of 10^5 c.f.u. on cotton dust themselves produced necrosis. However, almost equal protection was given by doses of 10^3 c.f.u. which did not.

IMMUNIZATION WITH COAGULASE-NEGATIVE STAPHYLOCOCCI AND WITH MICROCOCCI

Here the degree of protection was variable, depending both on the immunizing and challenge organisms (Table 2). M.278 protected mice against the three virulent *S. aureus*

TABLE 2

EFFECT OF IMMUNIZATION BY COAGULASE-NEGATIVE STAPHYLOCOCCI (10^5 c.f.u.) ON *S. aureus* LESIONS IN MICE

Challenge strain (10^5 c.f.u.)*	Immunizing strain*†									
	0		M278		*Micrococcus hyicus*		1061		1042	
	A	B	A	B	A	B	A	B	A	B
M278	0	0	—	—	—	—	—	—	—	—
PS80	12	38	2	1·7	2	1·0	10	13	8	9
Fisher	12	54	2	1·0	—	—	—	—	—	—
Orbach	12	40	0	0	—	—	0	0	0	0

* Virulent strains, *S. aureus* PS80, Fisher and Orbach; non-virulent strains, coagulase-negative staphylococci M278, *M. hyicus* 1061, 1042.
† A = number of mice with necrosis in a group of twelve; B = mean necrotic index for the group.

strains as effectively as immunization with *S. aureus*. Strains 1042 and 1061, on the other hand, were more effective against Orbach than against PS.80. Even with PS.80, however,

the number of immunized mice showing necrotic lesions was slightly reduced and the areas of necrosis were smaller.

OTHER METHODS OF IMMUNIZATION

Active immunization of mice with α-toxin and passive immunization with anti-α-toxin or with serum from previously infected mice all prevented dermonecrosis following challenge with virulent *S. aureus* (Table 3).

TABLE 3

EFFECT OF ANTI-INFLAMMATORY AGENTS ON *S. aureus* (10^5 c.f.u. OF PS80) LESIONS IN IMMUNE MICE

Type of immunization	Anti-inflammatory agent*							
	None		ATS		Prednisone		Zymosan	
	A	B	A	B	A	B	A	B
None	12	34	12	62	12	80	12	74
Live PS80†	0	0	12	15	12	17	12	11
Immune serum‡	0	0	7	7	10	20	10	8
anti-α-toxin	0	0	12	15	12	13	9	7

* A number of mice with necrosis in a group of twelve; B = mean necrotic index.
† Two previous infections with PS80.
‡ Serum from two previous infections with PS80.

THE EFFECT OF ANTI-INFLAMMATORY AGENTS AND COMPLEMENT DEPLETION ON DERMONECROSIS

When the early inflammatory response to staphylococcal infection was suppressed by ATS or prednisone or by complement depletion with zymosan, the frequency and intensity of dermonecrosis was increased (Table 4).

TABLE 4

EFFECT OF ANTI-INFLAMMATORY AGENTS ON *S. aureus* (PS80) LESIONS IN NON-IMMUNE MICE

Challenge dose (c.f.u.)	Anti-inflammatory agent*							
	None		ATS		Prednisone		Zymosan	
	A	B	A	B	A	B	A	B
10^4	2	0·1	7	9	10	40	12	55
10^5	12	34	12	62	12	80	12	74

* A = number of mice with necrosis in a group of twelve; B = mean necrotic index for the group.

Even in mice previously infected or given immune serum or anti-α-toxin, all procedures which normally provide excellent protection against necrosis, the use of anti-inflammatory agents resulted in necrotic lesions (Table 3).

THE EFFECT OF NON-SPECIFIC ACUTE INFLAMMATION ON STAPHYLOCOCCAL DERMONECROSIS

Glycogen injected subcutaneously produced a good local inflammatory response but no

TABLE 5

EFFECT OF GLYCOGEN ON *S. aureus* (10^5 c.f.u. PS80) LESIONS IN NON-IMMUNE MICE

Challenge strain	Control		Glycogen	
	A	B	A	B
PS80	12	42	3	2·5
Fisher	12	34	1	0·5

A = number of mice with necrosis in a group of twelve; B = mean necrotic index.

macroscopic necrosis. High doses (10^5 c.f.u. on cotton dust) of virulent staphylococci injected into such areas failed to produce necrosis (Table 5). When similar doses were injected into other sites in the same mouse, however, there was no reduction in lesion severity.

Finally staphylococci were injected subcutaneously together with anti-bovine serum albumin while bovine serum albumin (BSA) was given intravenously. The resulting passive reversed Arthus reaction was followed by a significant decrease in the necrosis due to *S. aureus* (Table 6). Some local inflammation occurred in controls given normal rabbit

TABLE 6

EFFECT OF IMMUNE INFLAMMATION ON *S. aureus* (STRAIN PS80, 10^5 c.f.u.) LESIONS IN MICE

Procedure	Early exudate (Evans blue)	Necrosis (No/12)	Necrotic Index
Anti-BSA s.c. +BSA i.v.	40	0	0
O	0	12	12·5
NRS s.c. +BSA i.v.	15	0	0
NRS s.c.	7	9	5·5
Anti-BSA s.c.	11	2	2

serum locally or BSA intravenously. Both reactions together gave slightly more inflammation though less than a full Arthus. The severity of the ultimate lesion was always inversely proportional to the degree of early inflammation produced.

TOXIGENICITY OF BACTERIAL STRAINS

None of the coagulase-negative strains produced detectable α-toxin on prolonged culture. No detectable antitoxin was detected in mice infected twice with such strains. Tested against rabbit red cells, culture filtrates from PS80 contained 64 international units/ml and from Wood 46, 1024 i.u. Infections with both the strains readily induced the production of anti-α-toxin.

DISCUSSION

All the virulent strains of staphylococci giving rise to dermonecrosis in the Noble–

Agarwal mouse model are producers of α-toxin. Even though Agarwal stressed the protective importance of early inflammation, he accepted the general view that necrosis was due to α-toxin and of course passively administered anti-α-toxin does prevent it. Although antibody neutralizes α-toxin both *in vitro* and *in vivo*, the experiments just described, together with previous work, suggest that this neutralization is less important than factors producing local inflammation. Thus although previous infection with strains PS80 or Wood 46 results in circulating anti-α-toxin and prevents necrosis, infection with coagulase-negative strains not toxigenic *in vitro* or *in vivo* is also protective.

Animals so immunized show an accelerated fluid exudation on challenge with staphylococci and do not develop necrotic lesions (Agarwal, 1967b). Goshi, Cluff and Norman (1963) found that rabbits gave a more intense cellular response to staphylococcal infection if they had been immunized with α-haemolysin. Hill (1969) protected mice with antibodies to DOCR and again a marked early inflammatory response was a feature. The local injection of sterile broth was as effective as the injection of staphylococcal culture filtrates in protecting against a staphylococcal infection according to Mallory and Marble (1925). Both agents caused local inflammation.

Similar results were obtained by Freedlander and Toomey (1928). These experiments, together with those of Miles *et al.* (1957) and our own results with glycogen, and the passive reversed Arthus reaction, all confirm the value of local inflammation. Injection of staphylococci into skin away from the inflamed area gave normal necrotic lesions. More significantly, where inflammation was suppressed necrosis was marked even though there was previously adequate circulating antibody.

The similarity of the results obtained with three different anti-inflammatory agents suggests that they are indeed due to the common anti-inflammatory action and not to the differing side effects. The regimes used would not be expected significantly to diminish antibody production in the time available and would not have affected passively administered anti-α-toxin. Nor, as used here, did the action of ATS depend on T-cell suppression.

From the experiments just described we suggest that immediate hypersensitivity has been due to a number of antibodies, with specificities against α-toxin, DOCR and as yet undetermined antigens in coagulase-negative staphylococci.

The latter at least seem unlikely to be involved in virulence and it looks as if one can dissociate the protective effect of inflammation from more specific effects of antibodies such as toxin neutralization. In the model used here the latter mechanism plays only a minor role. It is worth stressing once again that although the fluid and cells of the inflammatory response may destroy staphylococci by non-specific means such as phagocytosis, it is a specific immune reaction that starts the process.

In all this it should be noted that we are discussing dermonecrosis and not abscess formation, which may be a separate problem. It is interesting that Hill (1969), in his work on immunization with DOCR, did find protection against the formation of any lesion. Perhaps it is a matter of the degree of the inflammatory response invoked. So far we have seen no protection against abscess formation after six or seven courses of immunization with live, virulent bacteria.

In addition this particular model may be of significance when considering human infection where abscess formation rather than necrosis is the main feature. Rogers and Melly (1965) suggested that human disease may be the result of infection superimposed on a state of high humoral immunity. In the mouse immunization brings the natural history of staphylococcal skin disease much closer to that of infection in man.

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