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EXPERIMENTAL STAPHYLOCOCCAL INFECTION IN MICE

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ONE of the main difficulties in establishing suitable models of staphylococcal infection has always been the resistance of the skin to infection, necessitating the use of artificially high inocula. Elek and Conen (1957) and later James and Macleod (1961) overcame this problem by injecting staphylococci subcutaneously on a cotton suture. The presence of a foreign body potentiated the infection. Noble (1965) made the system more quantitative by using, as a foreign body, a pellet of cotton dust inserted into a wide-bore needle. The cotton dust, with an accurately measured number of staphylococci, could then be expelled subcutaneously with a wire plunger. In this system, virulence was defined on the basis of dermonecrosis at the injection site.

Using this model of staphylococcal infection, Agarwal (1967*a, b* and *c*) was able to show that there was an inverse relationship between the virulence of the staphylococcal strain and the amount of early fluid exudation and leucocyte infiltration. Repeated staphylococcal infection resulted in protection against dermonecrosis, but not against local bacterial multiplication and abscess formation. Anti-inflammatory agents increased the severity of infection. Of particular interest was the finding that some coagulase-negative strains could impede the development of the early acute inflammatory response although they did not produce dermonecrosis. This suggested that the full expression of staphylococcal virulence required both the capacity to suppress local defences in the skin and to produce tissue damage. Hill (1968) showed that a peptidoglycan "aggressin", which was left as the residue after cell walls from log-phase skin-virulent strains of *Staphylococcus aureus* had been treated with deoxychol-

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ate, not only acted as a local anti-inflammatory agent but also increased the virulence of avirulent strains of *S. aureus* when injected subcutaneously with them. Glynn (1972) suggested that the term "impedin" would be more appropriate for this virulence factor because it did not appear to be directly toxic. The deoxycholate residue (DOCR) was also a potent anti-inflammatory agent when the experimental animal was infected either intravenously or intraperitoneally. Work by Easmon, Hamilton and Glynn (1973) suggested that DOCR acted upon the kinin system by inhibiting the release of kinins. DOCR also impaired leucocyte chemotaxis *in vitro* (Weksler and Hill, 1969).

Staphylococcal virulence factors and the route of infection

When *S. aureus* is injected subcutaneously into mice, the major determinant of virulence is DOCR, which acts by preventing early local inflammation at the site of infection. Although the main effect of humoral immunity in this system, including of course antibody to DOCR, is to produce an accelerated acute inflammatory response, the specific neutralising action of antibodies to staphylococcal toxins may also play a part in modifying dermonecrotic lesions (Easmon and Glynn, 1975a). Even severe dermonecrotic lesions usually remain localised and with this model of infection it is very difficult to produce systemic spread of *S. aureus* and death.

In contrast, when the intraperitoneal route of infection is used, the major determinant of staphylococcal virulence is the presence of a polysaccharide capsule, which allows the capsulate strains of *S. aureus* to resist ingestion and killing by the macrophages and polymorphs of the peritoneal cavity (Koenig, Melly and Rogers, 1962; Melly *et al.*, 1974). Virulent *S. aureus* strains, i.e., those with capsules, multiply and eventually kill the mouse, possibly by the release of α haemolysin, whereas avirulent strains are phagocytosed and killed. In the peritoneal cavity, humoral immunity involves the production of anticapsular antibody which facilitates the ingestion and killing of the capsulate *S. aureus*. There are several capsular types, and humoral immunity is type specific (Melly *et al.*, 1974).

The differences between the two routes of infection are striking. Easmon and Glynn (1976) made a comparative study of strains of *S. aureus* that were virulent by either route of infection. *S. aureus* strain PS80, possessing DOCR, produced dermonecrosis when injected subcutaneously but had no effect when given intraperitoneally. *S. aureus* strains Smith diffuse and M, capsulate but without DOCR, killed mice when given intraperitoneally but failed to produce dermonecrotic lesions after subcutaneous injection (tables I and II). The capsulate strains grew no faster in subcutaneous tissue than did avirulent non-capsulate strains PS80 and Wood 46, suggesting that the antiphagocytic capsule is most effective in fluid-filled cavities where random contact between bacterium and phagocytic cell is less likely to occur than in the tissues.

Complement depletion increased the virulence of *S. aureus* strains in the skin and in the peritoneal cavity (tables III and IV). In the peritoneal cavity, neutrophil chemotaxis was reduced, as was opsonisation and intracellular

TABLE I

Virulence of Staphylococcus aureus strains injected subcutaneously into outbred Wright-Fleming Institute mice

Strain of <i>S. aureus</i>	Presence of		Necrotic index*	Viable count 10^5 c.f.u./ml ($\pm$ SD) at	
	DOCR	capsule		4 h	24 h
PS80	+	—	28	1.0 (0.15)	200 (40)
Wood 46	—	—	4	2.0 (0.3)	200 (38)
Smith diffuse	—	+	0	1.5 (0.1)	150 (27)
M	—	+	0	2.0 (0.2)	300 (41)

DOCR=anti-inflammatory residue after extraction of cell walls with deoxycholate; + = present; — = absent.

* Necrotic index = $\frac{\Sigma(Dd)}{n}$ where D and d = (respectively) major and minor diameter (mm) of necrotic lesion, and n = number of mice.

TABLE II

Virulence of Staphylococcus aureus strains injected intraperitoneally into mice

Strain of <i>S. aureus</i>	Presence of		Inoculum (c.f.u.)	Mortality (number dead per group of 20)
	DOCR	capsule		
PS80	+	—	10^9	8
			10^8	0
Wood 46	—	—	10^9	14
			10^8	0
Smith diffuse	—	+	10^8	20
			10^7	6
M	—	+	10^7	20
			10^6	0

Abbreviations as in table I.

killing. In the skin, complement depletion inhibited the cellular and the humoral components of the acute response, but had no effect on the killing of staphylococci (Easmon and Glynn, 1976). The experiments described in this section illustrate the importance of relating virulence and virulence factors to the route of infection and the bacterial strains used, as well as to bacterial and host species, especially for an organism such as *S. aureus* which causes infections in a wide variety of sites. The study of *S. aureus* infection in one site may have little relevance to infection in other sites.

Repeated staphylococcal infection and cell-mediated hypersensitivity

S. aureus infections are normally acute and associated with a localised polymorph response and a polymorph leucocytosis in the blood. A small group of patients, however, suffer from chronic or recurrent staphylococcal

TABLE III
Effect of complement depletion* on staphylococcal skin lesions in mice

Strain of <i>S. aureus</i>	Treatment of mice with CVF	Necrotic index†
PS80	—	25
	+	55
Wood 46	—	0
	+	20
Smith diffuse	—	0
	+	10
M	—	0
	+	15

CVF = Cobra-venom-factor; + = treated; — = not treated.

* Mice given two intraperitoneal injections of CVF and used 24 h later, when C3 CH50 were < 10% of pre-treatment levels.

† See footnote to table I.

TABLE IV
Effect of complement depletion* on mortality of mice after intraperitoneal infection with *Staphylococcus aureus*

Strain of <i>S. aureus</i>	Inoculum (c.f.u.)	Treatment of mice with CVF	Mortality (number dead per group of 20)
PS80	10 ⁸	—	0
	10 ⁸	+	20
Wood 46	10 ⁸	—	0
	10 ⁸	+	15
Smith diffuse	10 ⁷	—	6
	10 ⁷	+	18
M	10 ⁶	—	0
	10 ⁶	+	18

Footnotes as in table III.

disease characterised by the formation of granulomata or cold abscesses. In this latter group, cell-mediated hypersensitivity might be of significance in the pathogenesis of the disease process.

Panton and Valentine (1929) provided the first experimental evidence for the role of cell-mediated delayed hypersensitivity in the pathogenesis of staphylococcal disease. Repeated staphylococcal skin infection rendered rabbits more susceptible to skin challenge with low numbers of *S. aureus*. This was confirmed by Johnson, Cluff and Goshi (1961) who also demonstrated that the susceptible rabbits showed hypersensitivity to killed *S. aureus*.

Johanovsky (1958) was also able to show that rabbits rendered hypersensitive to *S. aureus* by repeated infection were more susceptible to subsequent staphylococcal challenge. Furthermore he found that this state of hypersensitivity could be passively transferred to non-infected recipients with lymphoid cells from sensitised donors but not with serum. Bøe (1945) explored

another facet of staphylococcal hypersensitivity by demonstrating, in rabbits and in man, that desensitisation by repeated intravenous injections of staphylococcal antigen improved chronic skin lesions. Taubler (1968) used a footpad-swelling test with *S. aureus* cell walls as antigen to show that mice too, after repeated infection, exhibited delayed hypersensitivity to staphylococcal antigen, which could be transferred to non-infected recipients with spleen cells from infected donors.

Easmon and Glynn (1975*b*), working with the model of staphylococcal infection developed by Noble (1965), found that, after four weekly staphylococcal skin infections, mice showed a delayed footpad response to *S. aureus* cell-wall antigen which could be suppressed by treatment with anti-thymocyte serum (table V). The delayed response was characterised by a mononuclear-cell infiltrate. Formal proof that this response was in fact T-lymphocyte mediated was obtained by the transfer of delayed hypersensitivity to non-infected recipients by spleen cells from sensitised donor mice and the prevention of this transfer by prior treatment of the spleen-cell suspension by anti-Thy 1 serum and complement (table VI). Thy 1 is an antigen found on

TABLE V

Effect of repeated skin infection with Staphylococcus aureus strain H on subsequent 24-h footpad reaction to the injection of 20 µg of homologous cell walls*

Number of skin infections	Footpad swelling	
	mm (± SD)	Significance (p)†
0	0.38 (0.03)	...
1	0.45 (0.03)	NS
2	0.41 (0.06)	NS
3	0.50 (0.08)	NS
4	0.93 (0.04)	<0.01
4 (with antithymocyte serum)	0.59 (0.07)	NS

* See Easmon and Glynn 1975*b*.

† Student's *t*-test; NS = not significant.

TABLE VI

Passive transfer of delayed hypersensitivity to Staphylococcus aureus strain H with spleen cells and its inhibition by the destruction of T cells by antithymocyte serum and complement

Donor mice	Treatment of transferred cells	Footpad swelling*	
		mm (± SD)	Significance (p)†
None	...	0.41 (0.04)	...
Non-infected	None	0.34 (0.05)	NS
Infected × 1	None	0.38 (0.07)	NS
Infected × 4	None	0.87 (0.05)	<0.01
Infected × 4	Antithymocyte serum + C'	0.48 (0.03)	NS

*† See footnotes to table V

murine T-cells (and nervous tissue), and treatment with antibody and complement lyses T cells (Reif and Allen, 1964).

Without proof of an effect on the staphylococcal lesion, the demonstration of cell-mediated hypersensitivity remains simply an immunological curiosity. We found that repeatedly infected mice that exhibited delayed hypersensitivity showed no change in the nature of their skin lesions. Serum from these mice protected non-infected mice from the dermonecrotic effects of *S. aureus* challenge but the transfer of spleen cells from the same infected donors increased the susceptibility of the recipients to staphylococcal challenge (Easmon and Glynn, 1975*b*). When serum and cells were transferred separately to the same recipient, the effect on subsequent staphylococcal challenge was the same as that of serum transfer (table VII). This implied not only that cell-mediated hypersensitivity to *S. aureus* is associated with increased susceptibility to staphylococcal skin infection, but also that this potentially

TABLE VII

Effect of cell and serum transfer from four-times infected donor mice on subsequent staphylococcal skin lesions in non-infected recipients

Transfer of		Necrotic index‡ (mean of 4 expts)
spleen cells*	serum†	
—	—	20
+	—	55
—	+	0
+	+	0

* 2×10^8 cells; + = transfer; — = no transfer.

† 0.3 ml.

‡ See footnote * to table I.

harmful response could be expressed only in the absence of the humoral response (Easmon and Glynn, 1975*b*).

Control of cell-mediated hypersensitivity to S. aureus in mice

The regulation or suppression of immune responses by immunocompetent cells or their products has attracted increasing interest in recent years. Lymphocytes (B and T), antibody, immune complexes and macrophages have all been shown to act in this way on humoral and on cell-mediated responses (Asherson and Zembala, 1976). The blocking of the effect of cell-mediated hypersensitivity by staphylococcal antibody or factors present in the serum of immunised mice suggested that the humoral response might have a regulatory effect on cell-mediated reactivity. Experiments were therefore designed to test this hypothesis.

These experiments centred on the use of the alkylating agent cyclophosphamide (CY) as a selective immunosuppressant. CY, itself an inert compound, is

converted by hepatic microsomal enzymes to a series of active metabolites which, having been incorporated into the DNA double helix, kill cells when they divide (Berenbaum, Cope and Double, 1973). CY therefore has a preferential effect on rapidly dividing cells. Dumont (1974) showed that CY depleted mice of B cells and a short-lived population of T cells, while Turk and Poulter (1972) found that, in guinea-pigs, the injection of CY led to a relative increase in the T-cell content of lymphoid tissue which was secondary to the depletion of the rapidly dividing B-cell population.

The administration of CY *before* antigen stimulation was found to enhance cell-mediated reactions when these were normally suppressed or regulated by rapidly dividing—and therefore highly susceptible—cells or their products (Turk, Parker and Poulter, 1972).

The intraperitoneal injection of CY 200 mg/kg into mice 2 days before a single subcutaneous injection of *S. aureus* did in fact enhance the delayed footpad response to *S. aureus* antigen to levels usually seen in mice only after four or more repeated staphylococcal infections (table VIII) (Easmon and

TABLE VIII

Effect of intraperitoneal cyclophosphamide (200 mg/kg) given 2 days before a single subcutaneous infection of 10^5 c.f.u. of Staphylococcus aureus strain H on the 24-h footpad response to 20 μ g of homologous cell walls

Preparation of mice		Footpad swelling*	
Treatment with CY	Number of infections	mm ($\pm$ SD)	Significance (p)†
—	0	0.37 (0.04)	NS
+	0	0.35 (0.03)	NS
—	1	0.46 (0.05)	...
+	1	1.13 (0.08)	<0.01
—	4	1.04 (0.10)	<0.01

CY = cyclophosphamide; + = treatment; — = no treatment.

*† See footnotes to table V.

Glynn, 1977). Cell-transfer experiments confirmed that this was a cell-mediated response. The enhancement of delayed hypersensitivity (DH) was seen only when CY was injected before staphylococcal infection in a dose of 100 mg/kg or more (tables IX, X). In addition to being a potent immunosuppressive, CY is an anti-inflammatory agent. It is therefore possible that the observed enhancement of DH was due to the persistence of antigen at the site of infection after the inhibition of inflammation by CY. Other anti-inflammatory agents, however, did not enhance DH to *S. aureus* antigens (Easmon and Glynn, 1977).

The enhancement of staphylococcal DH by pre-treatment with CY established that there must be regulatory mechanisms involved that normally prevent the emergence of this potentially harmful cell-mediated response and strongly suggest that repeated infection serves to override these controls.

TABLE IX

Effect of intraperitoneal cyclophosphamide (200 mg/kg) given at various times relative to infection with Staphylococcus aureus strain H on the 24-h footpad response to homologous cell walls

Day of CY treatment relative to infection	Footpad swelling*	
	mm ($\pm$ SD)	Significance (p)†
None given	0.35 (0.08)	0
-3	1.15 (0.07)	<0.01
-2	1.21 (0.11)	<0.01
-1	0.97 (0.07)	<0.01
0	0.79 (0.06)	<0.01
+1	0.58 (0.07)	NS
+2	0.41 (0.03)	NS
+5	0.40 (0.06)	NS

CY = cyclophosphamide.

*† See footnotes to table V.

TABLE X

Effect of various intraperitoneal doses of cyclophosphamide given to mice 2 days before infection with Staphylococcus aureus strain H on the 24-h footpad response to homologous cell walls

Dose of CY (mg/kg)	Sensitising infections of mice	Footpad swelling*	
		mm ($\pm$ SD)	Significance (p)†
0	-	0.38 (0.06)	NS
0	+	0.57 (0.08)	...
25	+	0.42 (0.08)	NS
50	+	0.41 (0.03)	NS
100	+	0.89 (0.07)	<0.02
200	+	1.10 (0.05)	<0.01
300	+	1.08 (0.05)	<0.01

CY = cyclophosphamide; + = sensitising infections given; - = not given.

*† See footnotes to table V.

In mice given one, two or three staphylococcal infections without CY treatment there must exist both active suppressive mechanisms and the potential, albeit suppressed, for DH. A series of experiments was therefore planned, in which either serum or cells were transferred from infected unresponsive mice into CY-treated infected donors (Easmon and Glynn, 1979). The aim was to look for the suppression of CY-enhanced DH to *S. aureus* by the passive transfer of humoral or cellular factors from mice in which DH was also being suppressed, and thus to analyse the mechanisms of suppression. Humoral suppression of DH was effective only when immune plasma or serum from mice given two or more *S. aureus* infections was transferred to CY-treated infected recipients within 3 days of the recipients' infection. Heating to 56°C for 30 min. had no effect on the suppressive effect of immune serum whereas either absorption with heat-killed *S. aureus* or treatment with dilute 2-mercaptoethanol abolished humoral suppression. The characteristics of cellular suppression were quite different, this being effective only when the cells were

transferred at or shortly before footpad challenge with antigen. The cells responsible were probably B lymphocytes, being non-phagocytic and bearing immunoglobulin-like surface receptors. Again, unlike humoral suppression, the cellular suppression of DH to *S. aureus* diminished sharply after three repeated infections, this waning of cellular activity coinciding with the appearance of DH to *S. aureus* in four-times infected mice (Easmon and Glynn, 1979).

The existence of more than one regulatory system acting on different aspects of an immune response has been well documented by the work of Asherson and Zembala (1976). Indeed, if the purpose of an immune regulatory system is to prevent harmful or useless responses, fine control could best be exercised by intervention at several points on any given pathway.

Antigens involved in delayed hypersensitivity

In all the experiments described above, a crude staphylococcal antigen preparation, washed cell walls, was used. Kowalski and Berman (1971) found that staphylococcal peptidoglycan and peptidoglycan fragments covalently linked to teichoic acid elicited DH in guinea-pigs that had been repeatedly infected with *S. aureus*, whereas teichoic acid and soluble low-molecular-weight peptidoglycan complexes did not. Staphylococcal protein A has also been shown to elicit DH reactions (Heczko, Grov and Oeding, 1973).

Easmon and Glynn (1978) used the CY system described earlier to investigate this problem. Peptidoglycan extracted from cell walls with dilute sodium hydroxide not only elicited DH in CY-treated infected mice in doses as low as 10 µg but also produced local dermonecrosis in sensitised mice (table XI). This

TABLE XI

Stimulation of delayed hypersensitivity in cyclophosphamide-treated mice by homologous cell-wall antigens

Sensitising infections	Staphylococcal antigen*	Footpad swelling† (mm) in mice that had been		Significance (p)‡
		CY treated	not CY treated	
—	Cell walls	0.38	0.34	NS
+	Cell walls	1.00	0.53	<0.01
—	Peptidoglycan	0.47	0.51	NS
+	Peptidoglycan	1.28§	0.64	<0.01
—	Teichoic acid	0.11	0.12	NS
+	Teichoic acid	0.23	0.15	NS
—	Protein A	0.05	0.08	NS
+	Protein A	0.19	0.09	NS

CY = cyclophosphamide; + = sensitising infections given; — = not given.

* *S. aureus* strain H cell walls disrupted and washed. Peptidoglycan and teichoic acid prepared by treatment of cell walls with dilute NaOH. For preparation of Protein A see Easmon and Glynn (1978).

† See Easmon and Glynn (1975b).

‡ Student's *t*-test.

§ Dermonecrosis.

observation related to our earlier finding that DH to *S. aureus*, in the absence of anti-staphylococcal antibody, was associated with severe dermonecrotic lesions when sensitised animals were challenged with viable staphylococci (Easmon and Glynn, 1975*b*). Protein A and teichoic acid did not stimulate DH and the cell-mediated response was species specific in that it could not be stimulated by infection with *S. epidermidis* or *S. saprophyticus* (Easmon and Glynn, 1978).

Although staphylococcal peptidoglycan appears to be the major antigenic determinant involved in cell-mediated reactions to *S. aureus*, the exact nature of this determinant is not known. It may prove possible to pursue this line of investigation either by splitting native peptidoglycan enzymically or by building up synthetic peptidoglycan analogues. Also unknown is the nature of the antigens that stimulate the regulatory mechanisms described above. An attractive hypothesis is that low-molecular-weight compounds released from the organisms during replication and destruction at the site of infection may act as tolerogens.

The significance of cell-mediated reactions to staphylococci in human infection

Animal experiments suggest that cell-mediated hypersensitivity to *S. aureus* can increase the severity of subsequent staphylococcal skin infection (Panton and Valentine, 1929; Johnson *et al.*, 1961; Easmon and Glynn, 1975*b*). Suppressive mechanisms normally control this potentially harmful immune response (Easmon and Glynn, 1977) which can, however, be overridden by persistent or recurrent staphylococcal infection (Easmon and Glynn, 1979).

If any parallels exist in human staphylococcal infection it would be logical to look for these in conditions in which there is persistence of staphylococcal antigen or viable cocci. Defects in polymorph chemotaxis (Hill *et al.*, 1974) and intracellular killing (Quie *et al.*, 1967) are associated with the formation of, respectively, cold abscesses and chronic granulomatous lesions. In both situations there is a failure of killing of the staphylococci; staphylococcal antigens therefore persist. The non-caseating granulomata are infiltrated with mononuclear cells and the picture is reminiscent of a cell-mediated inflammatory response.

In these conditions, as in the animal experiments, the high load of staphylococcal antigen, much of it intracellular, may lead to the stimulation of delayed hypersensitivity and to the overriding of the control mechanisms that normally keep in check this potentially damaging immune response. Much has been learnt about intracellular killing mechanisms by the study of chronic granulomatous disease. This syndrome may now be able to provide us with information about the role of immunological control mechanisms in bacterial disease.

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