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Cell-mediated Immune Responses in *Staphylococcus aureus* Infections in Mice

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Summary. Delayed hypersensitivity to staphylococcal antigens was shown in mice repeatedly infected with *Staphylococcus aureus*. It was characterized by footpad swelling at 48 hours with a mononuclear cell infiltrate and could be transferred to non-infected recipients by T lymphocytes from infected animals, but not by serum. Recipients of immune T cells produced very severe necrotic lesions when challenged with staphylococci. This was in contrast to the protection against necrosis in recipients afforded by serum from infected donors. When both serum and cells were transferred into the same mouse the humoral effects overshadowed or perhaps inhibited those mediated by cells with resultant protection against staphylococcal dermonecrosis.

INTRODUCTION

Although a considerable amount of work has been done on the immunology of staphylococcal infection, much of it has been concentrated on humoral and non-specific mechanisms. Less is known about the role of specific cell-mediated responses in the pathogenesis of staphylococcal disease. Panton and Valentine (1929) showed that, in rabbits, delayed hypersensitivity developed after repeated staphylococcal infections. In such hypersensitive animals the minimum inoculum producing a skin lesion was lowered. Johnson, Cluff and Goshi (1961) confirmed and expanded these findings using a series of intradermal staphylococcal infections. Their technique did not lead to the development of humoral immunity which simplified the interpretation of the cell-mediated responses. Johanovsky (1958) could transfer the hypersensitivity developing after repeated staphylococcal infection to other rabbits with living lymphocytes, but not with serum or dead cells. More recently Taubler (1968) and Taubler and Mudd (1968) have shown delayed hypersensitivity to staphylococcal antigens in mice *in vivo* and by inhibition of cell migration *in vitro* but did not investigate its effect on the lesions produced

Cellular responses to bacteria are mediated by thymus-dependent (T) lymphocytes. The effect of the transfer or depletion of these cells has generally been studied in infections where cell-mediated responses are a major factor, such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* in mice (Mackaness and Blanden, 1967; Lane and Unanue, 1972; North, 1973).

In this paper we have demonstrated the presence in infected mice of delayed hyper-

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sensitivity to staphylococcal antigens, and have used the techniques outlined above to investigate the effect of specific cellular responses on staphylococcal skin lesions.

MATERIALS AND METHODS

Mice

Female CBA mice 20–25 g weight and 'nude' mice between 12 and 16 weeks old were used.

Organisms

Staphylococcus aureus PS80 (NCTC 9789) was grown in 0·1 per cent glucose broth and injected subcutaneously. The details as well as the method of scoring the lesions are given in the previous paper (Easmon and Glynn, 1975).

Viable counts

Lesions were excised and homogenized in 10 ml of saline in a Colworth Stomacher 80 (A. J. Seward & Co Ltd). Ten-fold dilutions were prepared and viable counts performed on nutrient agar plates.

Fluid exudation

This was measured by the increase in weight of a constant sized piece of tissue containing the lesion (Agarwal, 1967a).

The induction of delayed hypersensitivity

Mice were given four subcutaneous injections of 10^5 c.f.u. of *S. aureus* PS80 on cotton dust at 10-day intervals. Twelve to 14 days after the last injection they were tested for delayed hypersensitivity by footpad swelling. Antigen (0·02 ml) was injected into the right hind footpad and the same volume of physiological saline into the left. The increase in footpad size was measured with a micrometer at 4, 24 and 48 hours. Net footpad swelling = swelling in right footpad − swelling in left footpad.

Uninfected mice were treated similarly and used as controls.

Antigen preparations

Saline extract. S. aureus PS80 in broth culture was spread over nutrient agar plates and incubated overnight at 37°. The total growth from ten plates was collected in 10 ml of sterile normal saline, washed twice in saline, and resuspended in a further 10 ml of saline. They were then put for 10 minutes under a Silverson mixer, centrifuged at 1000 ***g*** for 30 minutes, and the supernatant collected and sterilized by millipore filtration (0·45 μm). The protein content was estimated by Lowry's method and the concentration in the extract adjusted to 500 μg/ml.

Cell wall suspension. A 3-hour culture of *S. aureus* PS80 was grown at 37° in a 1 per cent nutrient broth (Oxoid number 2) with 1 per cent yeast extract (Oxoid) added with vigorous shaking, and was harvested and washed three times (10,000 ***g*** for 30 minutes) with normal saline. The cells were broken down in a Braun homogenizer with glass beads until no whole Gram-positive cells could be seen on staining. Whole cells and glass beads were separated by centrifugation at 1000 ***g*** for 15 minutes. The supernatant was centrifuged and washed twice in 1·5 M saline, three times in normal saline and three times in distilled

water each for 30 minutes at 10,000 ***g*** and then freeze-dried. Before each experiment this cell wall material was made up in saline to a concentration of 3 mg/ml.

Histology

Sections of footpads were taken at 4, 24 and 48 hours, fixed in formol saline and stained with haematoxylin and eosin.

Anti-thymocyte serum (ATS)

This was prepared by injecting CBA thymocytes into New Zealand white rabbits (Levey and Medawar, 1966).

ATS was inactivated and used unabsorbed in doses of 0·20 ml i.p. per mouse.

Anti-θ C3H serum

This was bought commercially (Searle Laboratories). It had been tested for cytotoxicity against CBA and AKR thymocytes by ^{51}Cr release. Its specificity was checked by absorption of its cytotoxic effect by CBA thymocytes.

Anti-θ C3H serum was used at a final dilution of 1:50. The source of complement was fresh guinea-pig serum which had been absorbed with agarose (Induboise A37 l'Industrie Biologique Française) to remove its innate toxicity for immune lymphocytes (Cohen and Schlesinger, 1970). For testing thymus, spleen and lymph node suspensions 0·05-ml volumes were used. The cells were suspended in Hanks's balanced salt solution, incubated with anti-θ serum, washed three times in veronal-buffered saline and then incubated with complement. In the cell transfer experiments 0·25 ml of anti-θ serum was used per 10^8 cells. Viability was estimated by trypan blue exclusion.

Depletion at T lymphocytes

At 6 weeks mice were thymectomized. Two weeks later they were given 300 mg/kg of cyclophosphamide (Cy) (Endoxana, Ward Blenkinsopp), and a further three doses of the same drug (200 mg/kg) at fortnightly intervals. Mice were tested for the presence of T cells by cytotoxic tests with anti-θ serum in cell suspensions.

Mice were used not less than 4 weeks after the last dose of cyclophosphamide.

In some experiments mice were reconstituted by the intravenous injection of 5×10^6 syngeneic thymocytes.

Transfer of lymphocytes from previously infected donors

Lymphocytes from mesenteric lymph nodes were harvested into Hanks's balanced salt solution by pressing the nodes gently through a fine wire mesh. The cells were washed three times in Hanks's solution + 10 per cent foetal calf serum and adjusted to a concentration of 2×10^8 cells per millilitre. Each recipient received 3×10^8 cells i.p. and was challenged with PS80 24–48 hours later. In some experiments cells were treated with anti-θ C3H serum, as described above, before transfer. Control mice received cells from non-infected donors.

Serum transfer

Mice received 0·2 ml of serum from previously infected donors immediately before infection with PS90.

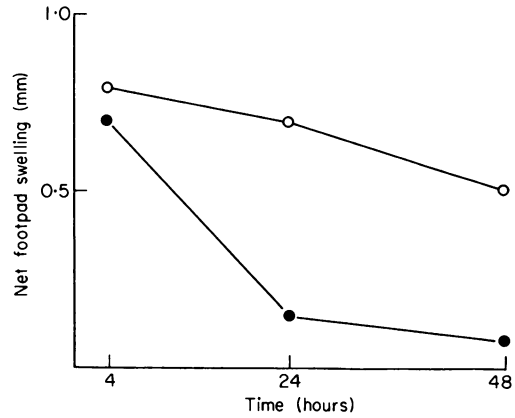

FIG. 1. Net footpad swelling induced by 60 μg of *S. aureus* PS80 cell wall suspension in infected (○) and non-infected (●) mice.

RESULTS

FOOTPAD REACTIONS IN NORMAL AND INFECTED MICE

Both antigen preparations gave a similar pattern of footpad swelling (Figs 1 and 2). At 4 hours there was some swelling in both infected and non-infected mice. This could be due to a non-specific irritant or possibly a reaction to protein A. At 24 and 48 hours the swelling persisted in the infected group only.

One group of infected mice was given ATS 3 days before footpad testing. The resultant swelling (Fig. 2) was reduced at 24 and 48 hours but was unaffected at 4 hours.

Persistent footpad swelling was also seen on testing normal mice who had been given lymph node cells from infected donors. Mice receiving immune serum from infected donors or lymph node cells from non-infected mice showed no delayed reaction (Fig. 3). Treatment of cell suspensions from the infected group with anti-θ C3H serum and complement removed their ability to transfer the delayed reaction.

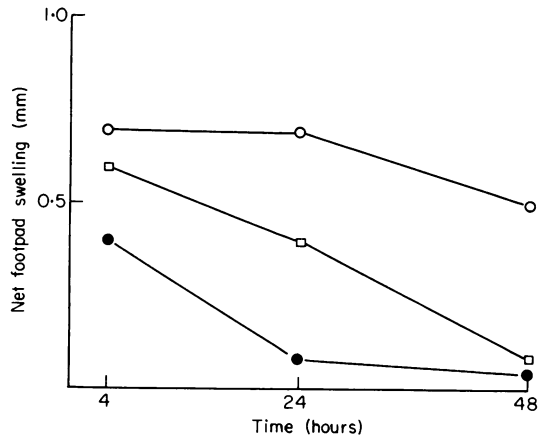

FIG. 2. Net footpad swelling induced by 0·02 ml of PS80 saline extract (500 μg protein/ml) in infected mice (○), infected mice pretreated with ATS (□) and non-infected mice (●).

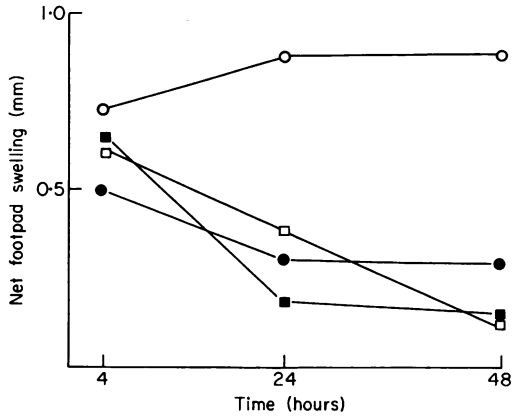

FIG. 3. The effect of cell and serum transfer on net footpad swelling in non-infected mice injected with: cells from infected donors (○); cells from infected donors treated *in vitro* with anti-θ C3H and complement (□); cells from non-infected donors (●); serum from infected donors (■).

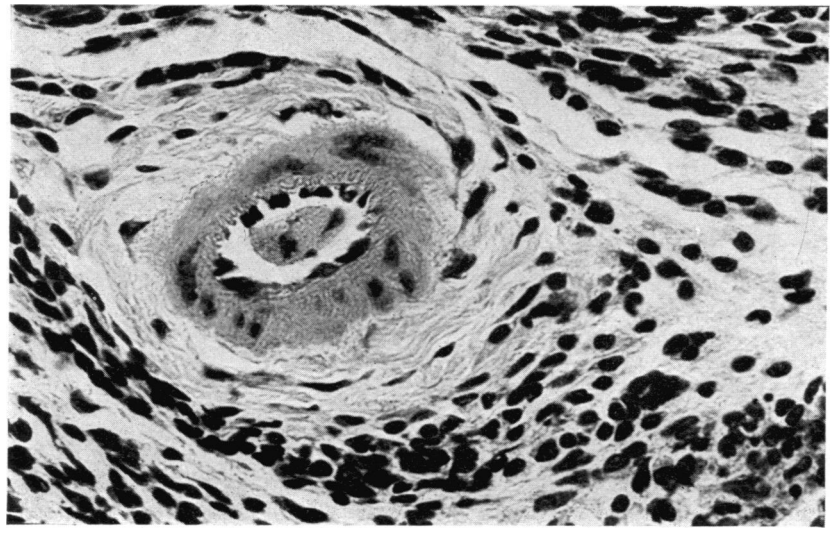

FIG. 4. Section of footpad swelling in an infected mouse 48 hours after the injected of PS80 cell walls, showing mononuclear infiltration. (Magnification ×350.)

Microscopy of the delayed reactions at 48 hours showed mononuclear infiltration (Fig. 4). In contrast the infiltrates in infected and non-infected groups at 4 hours (Fig. 5) and in the control group at 48 hours were predominantly polymorphonuclear.

STAPHYLOCOCCAL SKIN LESIONS IN NORMAL MICE AND AFTER TRANSFER OF LYMPH NODE CELLS FROM INFECTED MICE

The results of this series of experiments are summarized in Table 1.

As Agarwal (1967a) had shown, when a normal mouse was injected subcutaneously with 10^5 colony-forming units (c.f.u.) of *S. aureus* PS80 on cotton dust, the typical 24-hour lesion was a necrotic area of skin. Following repeated infections, delayed hypersensitivity appeared, and the lesions were milder, the necrosis being replaced by erythema and pus. The ability of serum from such animals to reduce the severity of lesions when given to primarily infected animals was reported by Agarwal (1967c) and dealt with more extensively in the preceding paper (Easmon and Glynn, 1975).

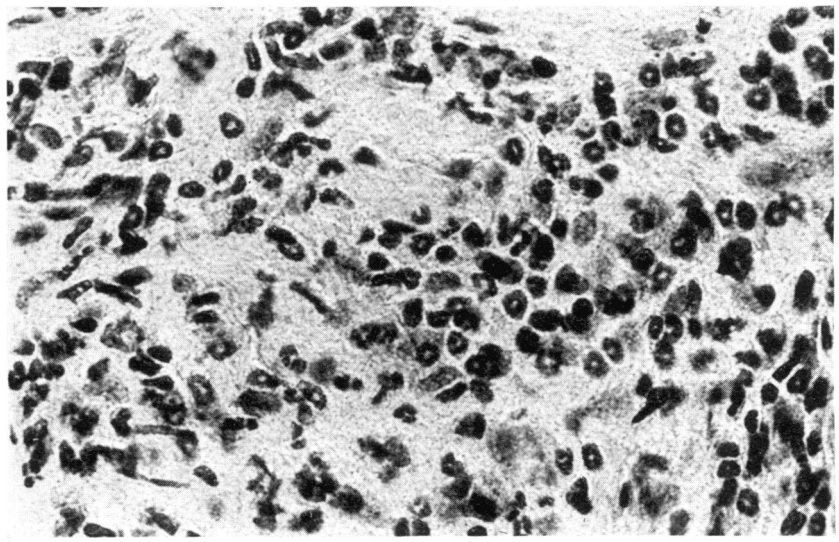

FIG. 5. Section of footpad swelling in a non-infected mouse 48 hours after the injection of PS80 cell walls. The infiltrate is predominantly polymorphonuclear. (Magnification × 350.)

When, however, living lymph node cells were transferred from infected mice and recipients challenged with *S. aureus* the lesions were severe with large areas of necrosis. This increase in severity was not seen when the donor cells were treated with anti-*θ* serum and complement before transfer. Lymph node cells from normal mice had no effect. In primarily infected mice given both immune cells and serum, the effect of the serum predominated and the lesions were mild and not necrotic.

STAPHYLOCOCCAL SKIN LESIONS IN MICE DEPLETED OF T CELLS

T cells in spleen and lymph nodes were measured by a cytotoxic test using anti-*θ* serum.

TABLE 1

Type of mouse	Number of mice with necrosis‡	Necrotic index
Non-infected	12	20
PS80 infected*	0	0
Given immune serum†	2	1
Given sensitized cells†	12	56
Given cells + serum	1	0·5
T cell-depleted	0	0
Nude mice	1	1
T cell-depleted and given syngeneic thymocytes	7	13

* Infected four times with PS80.
† Serum and cells from mice infected four times with PS80.
‡ Groups of twelve mice.

In the thymectomized mice treated with cyclophosphamide as described the spleen and lymph nodes contained less than 10 per cent of T cells compared with 28 per cent and 50 per cent respectively in normal mice. In these T cell-depleted mice the staphylococcal lesions were mild with no necrosis. This was despite the fact that the mice had undergone thymectomy and severe chemotherapy which might have been expected to reduce resistance to infection generally. If the mice were reconstituted with normal syngeneic thymocytes then necrotic lesions were seen on infection, as in normal mice.

Staphylococcal lesions were mild and not necrotic in 'nude' mice in which there are very few T cells.

BACTERIAL COUNTS IN STAPHYLOCOCCAL SKIN LESIONS

The numbers of viable bacteria found at different times in the lesions in normal and previously infected mice were very similar, although the lesions were very different (Fig. 6). Bacterial counts from mice given normal or immune serum, or lymphocytes from normal or infected animals were almost identical.

Agarwal (1967b, c) found differences in the pattern of growth between non-infected and previously infected mice, using an inoculum of 10^3 c.f.u. He did, however, choose this lower dose because with higher inocula he found differences between the groups less obvious.

T CELLS AND FLUID EXUDATION

Fig. 7 shows the early fluid exudation of four groups of mice in response to infection with *S. aureus* PS80. As already described (Agarwal, 1967b; Easmon, Hamilton and Glynn, 1973) in the normal group there was a delay of 1 hour before exudation became apparent, while in the previously infected group the exudate was greater and appeared earlier. In the T cell-depleted group the early exudation was the same as in normal uninfected mice. In the mice given lymphocytes, however, the degree of fluid exudation was smaller than in the non-infected controls and even further delayed.

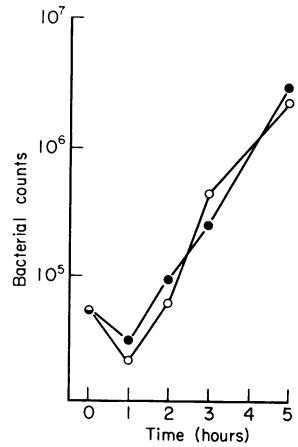

FIG. 6. Viable bacterial counts in *S. aureus* PS80 lesions from infected (○) and non-infected mice (●). Initial inoculum 10^5 c.f.u.

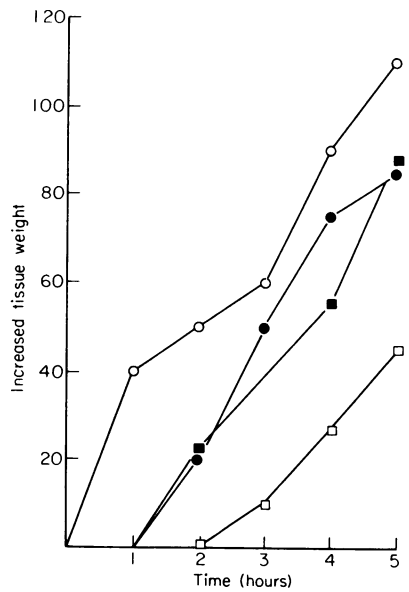

FIG. 7. Amount of early fluid exudate following infection with *S. aureus* PS80 in: normal mice (●); previously infected mice (○); T cell-depleted mice (■); mice given immune lymphocytes (□).

DISCUSSION

Delayed hypersensitivity to *S. aureus* in mice is not a new finding but its significance in the pathogenesis of infections is not clear. The experiments just described show that local staphylococcal lesions are characterized by severe necrosis. Following repeated staphylococcal infections delayed hypersensitivity to crude staphylococcal antigens is detectable. Both these reactions are attributed to cell-mediated immune responses because of their timing, the histology of the footpads and because both can be induced following adoptive

transfer of immune T cells. The experiments with nude mice confirm the importance of T cells.

When both immune cells and antibody were present the humoral effect predominated and lesions were mild and not necrotic. This may explain why repeated skin infections in mice did not lead to increased susceptibility to staphylococci, such as was found in rabbits by Johnson *et al.* (1961). In their model of infection no humoral response was observed.

From Agarwal's early work (Agarwal, 1967b) the general rule was derived that in local staphylococcal infection the severity of the late (24–48-hour) lesion was inversely proportional to the intensity of the early (<6 hours) inflammation. In experiments (Agarwal 1967b, c) with inocula of 10^3 *S. aureus* it was found that the viable counts in lesions at 24–48 hours were lower in immune than in normal mice, suggesting that the increased early inflammation had killed more bacteria. However, both Agarwal and ourselves found no such effect of inflammation when the inoculum was increased to 10^5 although there was still less late necrosis in immune mice compared with normal mice. The protection found in immune mice cannot, therefore, be attributed to increased killing of bacteria. If it is accepted that the necrosis is due to delayed hypersensitivity then it is possible that in immune mice the extra polymorph activity consequent on early active inflammation destroys the relevant antigens without affecting the bacterial viable count, or that the antigen reacts with antibody which thus blocks the delayed hypersensitivity response. The latter is perhaps more likely and would be an infectious parallel of the inhibition by antibody of cell-mediated immunity to tumours seen in the facilitation–enhancement reaction (Voisin, 1971). Lagrange, Mackaness and Miller (1974) have recently stressed the complex inhibitory role of antibody on delayed type hypersensitivity. However, on this basis it is difficult to explain the effect of non-immune inflammation such as follows glycogen. Possibly a simple dilution effect of exudate also plays a part.

In the current model of cell-mediated immunity, specifically stimulated lymphocytes induce hyper-phagocytic and bactericidal activities in macrophages which protect against many chronic intracellular infections (Mackaness and Blanden, 1967). This is in marked contrast to the harmful effect of cell-mediated immunity in our experiments. As the work of Agarwal (1967a, b and c) shows, the outcome of a staphylococcal infection is decided by the early inflammatory events, that is before active macrophages have time to play a part. Nor is there any evidence that staphylococci can survive in macrophages as intracellular parasites even in chronic infections. Nevertheless staphylococcal infection can produce active macrophages. In rabbits Lenhart and Mudd (1971) showed that the macrophages in animals repeatedly infected with *S. aureus* and activated with a staphylococcal phage lysate had an increased capability to kill staphylococci. Shayegani, De Courcy and Mudd (1973) have produced similar results in mice. Allen and Mudd (1973) have shown that repeated staphylococcal infection can protect mice against infection with vaccinia.

We depleted our mice of T cells with a combination of adult thymectomy and cyclophosphamide, rather than by using lethal irradiation and marrow reconstitution. Cyclophosphamide has been used on its own to deplete preferentially short-lived B lymphocytes, on the basis that they have greater mitotic activity and will, therefore, be more susceptible (Lennan and Weidang, 1970; Turk and Poulter, 1972). This is only a relative effect and Stockman, Heim and Trentin (1973) and Winklestein (1973) have found that cyclophosphamide can depress T-cell function, as measured by the PHA response. Jokipii and Jokipii (1973) depressed the delayed hypersensitivity reaction to tuberculous PPD. Using

adult thymectomy and one single dose of cyclophosphamide Ainsberg and Murray (1971) did not find significant T-cell depression. In mice given a single dose of cyclophosphamide there was a 5-fold reduction in numbers of the long-lived T cells and an even greater reduction of the short-lived T and the B cells. Regeneration of all types of cell was complete in 30 days (Dumont, 1974).

We have, however, used four doses of this drug given at intervals where the cells might be expected to be recovering from the effects of the previous dose and therefore at their most sensitive. If T cells are killed in a thymectomized animal they cannot be replaced. There seems to be no advantage over irradiation in either time, morbidity, or mortality, in the use of cyclophosphamide, but used in this way it was effective as judged by cytotoxic tests with anti-θ serum. Challenge of these mice with staphylococci can be considered a very severe test. Impairment of B-cell capacity in an infection where humoral immunity appears to be protective could result in very severe lesions. In fact, as already described, the lesions were milder than those in the controls. The later experiments on the 'nude' mice which have very few T cells gave similar results. Further investigation of the effects of subpopulations of T cells is indicated. One finding which is difficult to explain is the reduction in the amount of early inflammatory exudate in mice given immune lymphocytes.

Indirectly our results contribute to the idea that the direct toxic action of staphylococcal α-haemolysin on tissue may have little to do with the production of necrotic lesions by virulent living organisms. In other experiments, not reported here, we found that the subcutaneous injection of α-toxin into normal and hypersensitive mice produced lesions of similar severity. Hypersensitive tissue was not unduly susceptible to the action of α-toxin and therefore the increase in lesion severity seen in hypersensitive mice challenged with living organisms was, presumably, not due to its action.

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REFERENCES

AGARWAL, D. S. (1967a). 'Subcutaneous staphylococcal infection in mice. I. The role of cotton dust in enhancing infection.' *Brit. J. exp. Path.*, **48,** 436.

AGARWAL, D. S. (1967b). 'Subcutaneous staphylococcal infection in mice. II. The inflammatory response to different strains of staphylococci and micrococci.' *Brit. J. exp. Path.*, **48,** 468.

AGARWAL, D. S. (1967c). 'Subcutaneous staphylococcal infection in mice. III. The effect of active and passive immunisation and anti-inflammatory drugs.' *Brit. J. exp. Path.*, **48,** 483.

AINSBERG, A. C. and MURRAY, C. (1971). 'The contrasting effects of cyclophosphamide and radiation on the immune responses of the mouse.' *J. exp. Med.*, **133,** 877.

ALLEN, E. G. and MUDD, S. (1973). 'Protection of mice against vaccinia virus by bacterial infection and sustained stimulation with specific bacterial antigens.' *Infect. Immun.*, **7,** 62.

COHEN, A. and SCHLESINGER, M. (1970). 'Absorption of guinea pig serum with agar. A method for elimination of its cytotoxicity for murine thymus cells.' *Transplantation*, **10,** 130.

DUMONT, F. (1974). 'Destruction and regeneration of lymphocyte populations in the mouse spleen after cyclophosphamide treatment.' *Int. Arch. Allergy*, **47,** 110.

EASMON, C. S. F. and GLYNN, A. A. (1975). 'The role of humoral immunity and acute inflammation in protection against staphylococcal dermonecrosis.' *Immunology*, **29,** 67.

EASMON, C. S. F., HAMILTON, I. and GLYNN, A. A. (1973). 'Mode of action of a staphylococcal anti-inflammatory factor.' *Brit. J. exp. Path.*, **54,** 638.

JOHANOVSKY, J. (1958). 'Role of delayed hypersensitivity in staphylococcal infections.' *Nature (Lond.)*, **182,** 1454.

JOHNSON, J. E., III, CLUFF, L. E. and GOSHI, K. (1961). 'Studies on the pathogenesis of staphylococcal infection. I. The effect of repeated skin infections.' *Brit. J. exp. Path.*, **113,** 235.

JOKIPII, A. M. M. and JOKIPII, L. (1973). 'Suppression of cell-mediated immunity by cyclophosphamide. Its independence of concomitant B cell response.' *Cell. Immunol.*, **9,** 477.

LAGRANGE, P. H., MACKANESS, G. B. and MILLER, T. E. (1974). 'Potentiation of T-cell mediated immunity by selective suppression of antibody formation with cyclophosphamide.' *J. exp. Med.*, **139,** 1529.

LANE, F. C. and UNANUE, E. R. (1972). 'Requirement of thymus (T) lymphocytes for resistance to listeriosis.' *J. exp. Med.*, **135,** 1104.

LENHART, N. and MUDD, S. (1971). 'Staphylococcid capability of rabbit peritoneal macrophages in relation to infection and elicitation: induction and elicitation of activated macrophages.' *Infect. Immun.*, **5,** 763.

LENNAN, S. P. and WEIDANG, W. P. (1970). 'The effects of cyclophosphamide on the ontogeny of the humoral immune response in chickens.' *J. Immunol.*, **105,** 614.

LEVEY, R. H. and MEDAWAR, P. B. (1966). 'Nature and mode of action of antilymphocyte sera.' *Proc. nat. Acad. Sci. (Wash.)*, **56,** 1128.

MACKANESS, G. B. and BLANDEN, R. V. (1967). 'Cellular immunity.' *Progr. Allergy*, **11,** 89.

NORTH, R. J. (1973). 'Importance of thymus derived lymphocytes in cell mediated immunity.' *Cell. Immunol.*, **7,** 166.

PANTON, P. N. and VALENTINE, F. C. O. (1929). 'Staphylococcal infection and reinfection.' *Brit. J. exp. Path.*, **10,** 257.

SHAYEGANI, M., COURCY, S. J. DE and MUDD, S. (1973). 'Cell mediated immunity in mice infected with *S. aureus* and elicited with specific bacterial antigens.' *J. reticuloendothel. Soc.*, **14,** 44.

STOCKMAN, G. D., HEIM, R. L. and TRENTIN, J. J. (1973). 'Differential effects of cyclophosphamide on the B and T cell compartments of adult mice.' *J. Immunol.*, **110,** 277.

TAUBLER, J. H. (1968). 'Staphylococcal delayed hypersensitivity in mice. I. Induction and *in vivo* demonstration of delayed hypersensitivity.' *J. Immunol.*, **101,** 546.

TAUBLER, J. H. and MUDD, S. (1968). 'Staphylococcal delayed hypersensitivity in mice. II. *In vitro* demonstration and specificity of delayed hypersensitivity.' *J. Immunol.*, **101,** 550.

TURK, J. L. and POULTER, L. W. (1972). 'Selective depletion of lymphoid tissue by cyclophosphamide.' *Clin. exp. Immunol.*, **10,** 285.

VOISIN, G. A. (1971). 'Immunological facilitation, a broadening of the concept of the enhancement phenomenon.' *Progr. Allergy*, **15,** 328.

WINKLESTEIN, A. (1973). 'Differential effects of immunosuppressants on lymphocyte function.' *J. clin. Invest.*, **52,** 2293.