SUBCUTANEOUS STAPHYLOCOCCAL INFECTION IN MICE*

III. EFFECT OF ACTIVE AND PASSIVE IMMUNIZATION AND ANTI-INFLAMMATORY DRUGS

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ALTHOUGH both immunity and hypersensitivity are known to develop in animals after staphylococcal infection (Panton and Valentine, 1929; Johanovský, 1958; Johnson, Cluff and Goshi, 1961), no detailed information is available about the effect of these changes on the inflammatory response to a new staphylococcal infection. In view of the importance of early inflammatory response in determining the final outcome of staphylococcal infection (Agarwal, 1967*a*, *b*), it was decided to examine the rate of exudation of oedema fluid, the rate of leucocyte emigration and the growth of cocci in the lesions of mice that had had a previous staphylococcal infection or had been treated with serum from " previously-infected " mice. The effect of some anti-inflammatory agents on the 2 components of staphylococcal inflammation and lesion production has also been examined.

MATERIALS AND METHODS

The staphylococcal strains used, the mice and the method of producing subcutaneous lesions were the same as in the work already described (Agarwal, 1967*a*).

Anti-inflammatory drugs.—The drugs used are listed in Table I; the doses shown in the Table were injected in 0·1 ml. volumes. All the drugs were dissolved in sterile double-distilled water, except Indomethacin which is not soluble in distilled water and for which sodium bicarbonate solution was used. A 1 per cent solution of sodium bicarbonate was added drop by drop to the powder until it dissolved; the requisite concentration of drug was made by adding sterile double-distilled water. The pH was checked and was kept between 8–9. The drug precipitates out when the solution has a pH value below 8.

Endotoxin treatment.—" Bacto " lipopolysaccharide from *Escherichia coli* 0 : 128 : B12 (Difco Laboratories) was used. A stock solution containing 1 mg. of lipopolysaccharide powder per ml. of sterile saline (0·9 per cent) was prepared and stored at — 20° C. Suitable dilutions were made in saline for injection. The dose of endotoxin was 0·5 μg./g. body weight and it was given i.v. Immediately afterwards the mice were challenged by the s.c. injection of cocci mixed with cotton.

Transfer of serum and lymph-node cells.—Serum from mice previously infected with a homologous strain of *Staphylococcus aureus* was collected and 0·25 ml. injected i.v. into normal mice. The injection was given immediately before challenge.

Lymph-node cells from passive transfer were prepared from mice " previously-infected " with *Staph. aureus* strain PS80. After killing the mouse, the lymph nodes were removed aseptically from cervical, axillary, inguinal, anterior abdominal and mesenteric areas. The lymph nodes were freed of fat and put in cold sterile Hanks' solution (Oxoid) containing 1 per cent lactalbumin hydrolysate.

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TABLE I.—*Anti-Inflammatory Drugs Used*

Drug	Dose μg./g.	Route	No. of injections
Colchicine	1	S.c.	S
Phenylbutazone sodium (" Butazolidin " Geigy)	240	S.c.	S
Chlorpromazine hydrochloride (" Largactil " May and Baker)	1	S.c.	S
Quinine hydrochloride	160	S.c.	S
Indomethacin (" Indocid ", Merck, Sharp and Dohme)	10	I.v.	S*
Cortisone acetate (" Cortisyl ", Roussel Laboratories)	125	I.m.	4D
Prednisolone disodium phosphate (" Predsol ", Glaxo)	100	I.p.	S
Dexamethasone-21-phosphate disodium salt (" Decadron ", Merck, Sharp and Dohme)	2	I.m.	4D
Betamethasone disodium phosphate salt (" Betnesol ", Glaxo)	8	I.m.	4D

S = Single dose given ½ hr. before the injection of cocci.
S* = Single dose given immediately before the inoculation of cocci.
4D = 4 daily doses, the last dose given 2 hr. before the injection of cocci.

The lymph nodes were teased between 2 pairs of forceps. The suspension was freed of cell debris by passage through fine nylon and the cells collected by centrifugation in a M.S.E. angle centrifuge at 2000 r.p.m. for a few min. The pad of cells was resuspended in an appropriate volume of Hanks' solution and the total number of cells counted in a haemocytometer chamber. In practice all the lymph node cells of 1 mouse were injected i.v. into a normal mouse. The number of cells injected varied between 7×10^7 and $1{\cdot}2 \times 10^8$ per mouse. The interval between killing the donor mice and injecting their cells into normal mice was never more than 4 hr. After 18 hr. the treated mice were injected s.c. with *Staph. aureus* strain PS80 mixed with cotton-dust.

RESULTS

The response of " previously-infected " mice to staphylococcal infection

Mice that had had 2 or 3 subcutaneous abscesses following injection of *Staph. aureus* strain PS80 in cotton-dust, were used. The interval between successive injections was 1–2 weeks. About 4 weeks after the last injection, when the lesions had completely healed with scar formation, the mice were used for these investigations. They were reinjected with 10^5 staphylococci of strain PS80 mixed with cotton-dust; care was taken to avoid injection into the scar tissue.

Lesion score.—In the " previously-infected " (PI) mice given a dose of 10^5 PS80, the lesions were mild, consisting mainly of swelling and pus formation. Only an occasional mouse showed a small zone of necrosis, 2–3 mm. in diameter. The average lesion score was 2·6. This value was based on the mean from 18 mice used in 3 different experiments. In contrast, control mice always showed severe lesions which were characterized by necrosis and pus formation. The average lesion score varied between 4·2 and 4·8. The reduction in the lesion score in the PI mice was mainly due to partial neutralization of skin necrosis.

No evidence could be obtained that repeated staphylococcal infection was associated with increased severity of the lesions, as might be expected if hypersensitivity were important. Twelve mice were injected with *Staph. aureus* strain PS80 mixed with cotton, at weekly intervals for 7 weeks. The average lesion score after each inoculation is shown in Table II. In none of the repeat injections was the lesion more severe than after the first injection.

TABLE II.—*Lesion Score After Repeated Injection of* Staph. aureus *Strain PS*80

No. of injection	Dose of PS80	Average lesion score/mouse
1	$5{\cdot}75\times10^5$	4·0
2	$3{\cdot}5\times10^5$	3·4
3	$3{\cdot}25\times10^5$	2·7
4	$1{\cdot}5\times10^5$	3·4
5	$5{\cdot}6\times10^5$	2·7
6	$9{\cdot}25\times10^5$	2·9
7	$6{\cdot}75\times10^5$	2·9

Fluid exudation.—The effect of a previous infection on fluid exudation in response to staphylococcal challenge is shown in Fig. 1. The controls were a group of mice that had been injected with cotton and sterile nutrient broth 4 weeks previously; they were reinoculated at the same time and with the same dose of PS80 as the PI mice. In the PI mice there was an early exudation of fluid, which was evident ½ hr. after the injection. Thereafter the fluid exudation went on

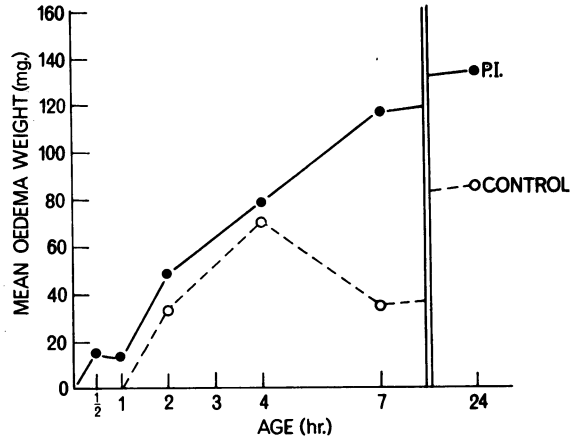

FIG. 1.—The mean oedema weight of tissue after the s.c. injection of *Staph. aureus* PS80 mixed with cotton dust in PI and control mice. Each point on the graph is the mean value from 4 mice.

increasing and at 24 hr. there was a considerable amount of oedema. In contrast, in the control mice, the fluid exudation started after an initial delay of 1 hr., and at 7 and 24 hr. the oedema was much less than in PI mice. No control mice were examined ½ hr. after inoculation; however, the injection of cotton and PS80 in normal mice had never elicited any exudation at ½ hr. in previous experiments (Agarwal, 1967*b*). Although the mean value for each point on the graph is based on only 4 mice, fluid exudation was evident at both ½ hr. and 1 hr. in 3 out of 4 PI mice. Furthermore on 2 other occasions mice that had experienced previous subcutaneous staphylococcal infection showed a similar response to a second injection, but in these experiments no parallel controls were included.

Leucocyte emigration.—The PI mice also responded with an accelerated leucocyte response (Fig. 2). Leucocyte emigration at the injection site was noticeable

at 1 hr. and infiltration of the cotton began at 3–4 hr.; by 7 hr. the entire cotton-dust was infiltrated by leucocytes. On the other hand in the control mice, emigration of leucocytes was not seen until after 2 hr. and these leucocytes remained at some distance from the edge of the cotton up to 4 hr. It was 7 hr. before any significant infiltration was seen and even at 24 hr. the central parts of the cotton-dust remained free of leucocytes.

Bacterial counts.—For the bacterial counts, 10^3 viable units of PS80 were injected and the results are shown in Fig. 3. The upper point on the ordinate

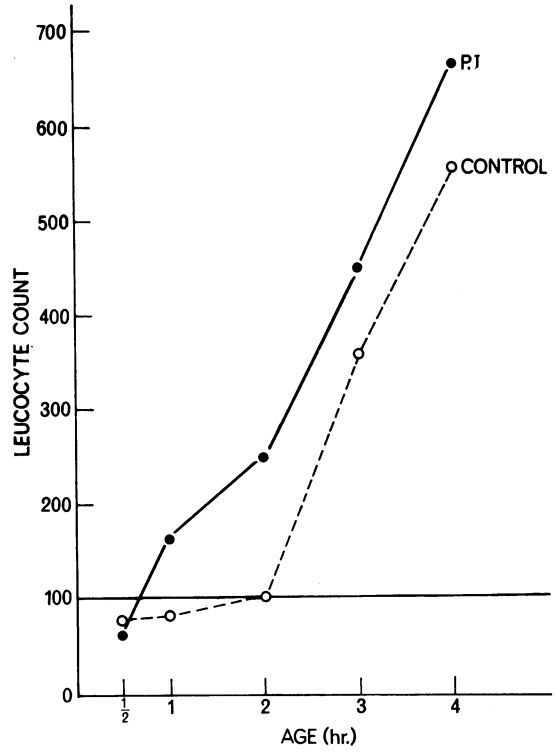

FIG 2.—Leucocyte counts in the lesions of PI and control mice after the s.c. injection of *Staph. aureus* PS80 mixed with cotton-dust.

shows the number of cocci injected and the lower point represents the " zero-hr " count, obtained by killing the animal and examining the lesion immediately after injection. In the PI mice the initial drop in the count was more and persisted for a longer period than in the controls. By 4 hr., the count had started to rise but even at 7 hr. it was lower than the inoculum. At 24 hr. the count was lower than at 7 hr.

Response to the injection of heat-killed Staph. aureus PS80

Previously-infected mice were injected with heat-killed *Staph. aureus* PS80 mixed with cotton-dust and their response was compared with that of normal mice also injected with heat-killed PS80 and cotton (Fig. 4). In the PI mice, there

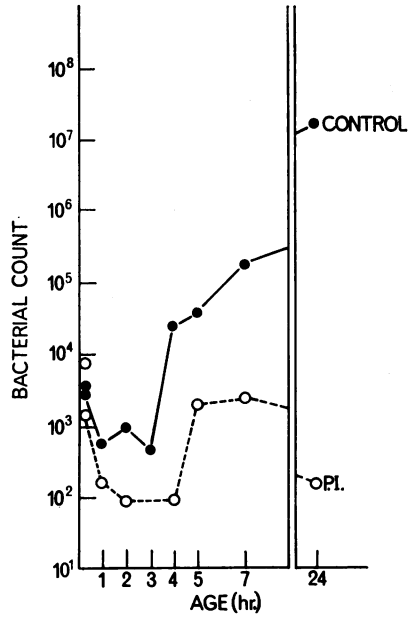

FIG. 3.—Bacterial counts in the lesion after the s.c. injection of *Staph. aureus* PS80 mixed with cotton-dust in PI and control mice. Each point represents the mean of the results taken from 4 mice.

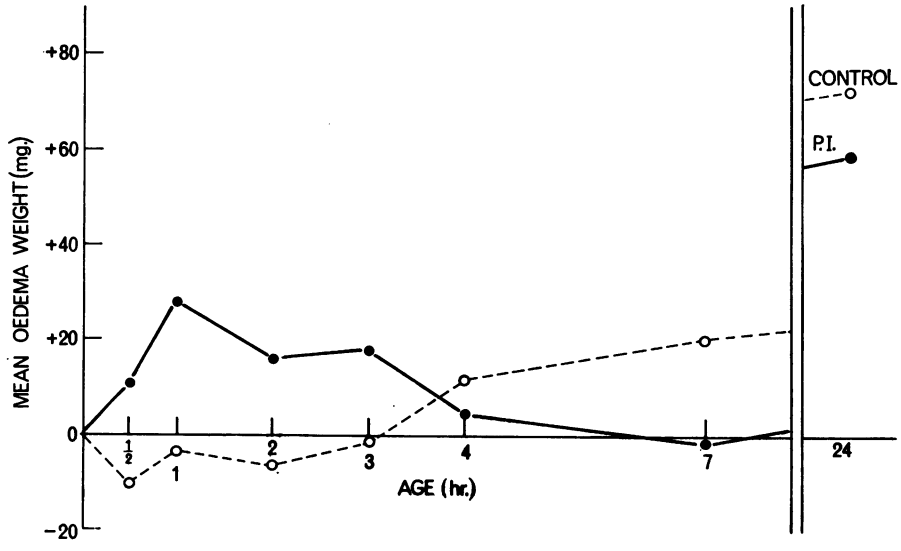

FIG. 4.—The mean oedema weight of tissue after the s.c. injection of heat-killed *Staph. aureus* PS80 mixed with cotton-dust in PI and control mice. Each point on the graph is the mean value from 4 mice.

was some exudation of fluid ½ hr. after the injection and during the first 3 hr. after injection, almost all the mice showed local exudation of fluid. In the control mice, no increase in tissue weight was discernible until after 3 hr.

In the PI mice, the emigration of leucocytes was evident at ½ hr.; the cotton itself was infiltrated at 3 hr. and by 24 hr. it was heavily infiltrated by leucocytes.

Response to the injection of α-haemolysin

" Wellcome " α-haemolysin (0·16 u.) was injected s.c. mixed with cotton-dust into PI and control mice (Fig. 5). The degree of fluid exudation tended to be

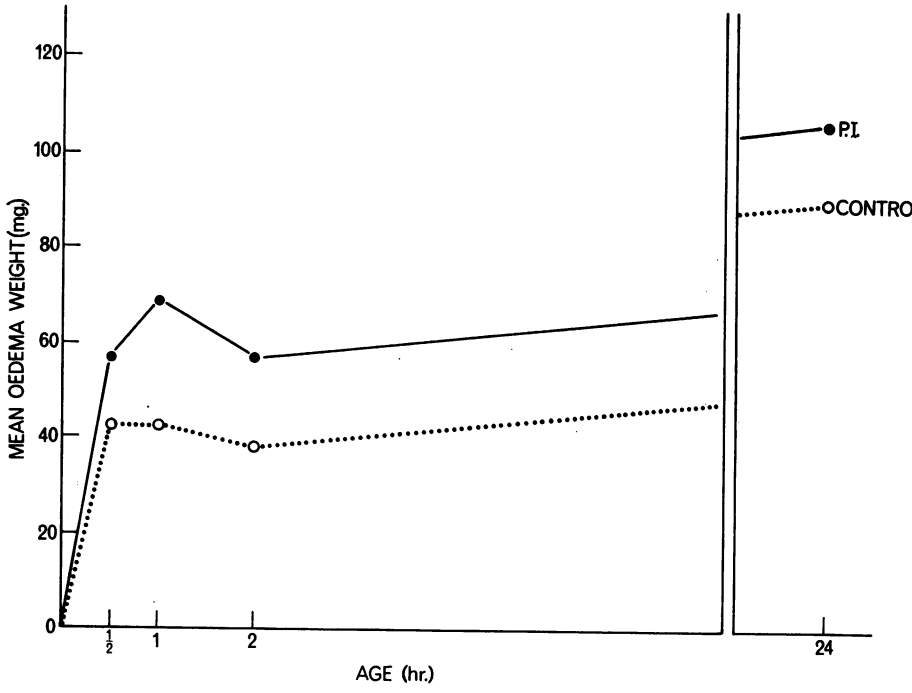

FIG. 5.—The mean oedema weight of tissue after the s.c. injection of " Wellcome " α-haemolysin with cotton-dust in PI and control mice. Each point on the graph is the mean value from 4 mice.

greater, both in the early stages and at 24 hr. in the PI mice than in the mice that had no past experience of staphylococcal infection.

Histological examination of the tissue revealed that in the PI mice the emigration of leucocytes was detectable by ½ hr., and infiltration of cotton 2 hr., after inoculation; by 24 hr. there was a massive infiltration of the entire cotton-dust. On the other hand, in the control mice, in spite of early emigration of leucocytes, the cotton was not infiltrated to any significant degree before 7 hr. and even at 24 hr. only the peripheral parts of the cotton were infiltrated.

PI mice showed evidence of immunity to dermonecrotic effects of α-haemolysin. While the control mice showed on average a zone of necrosis 3·1 mm. in diameter; in most cases the PI mice had no necrosis or only rarely a pin-point area of necrosis.

Passive transfer experiments

Serum transfer.—In the previous section, it was shown that mice with past experience of staphylococcal infection exhibited some degree of protection and an accelerated inflammatory response to the injection of *Staph. aureus* PS80 and α-toxin. It is also known that in the local anaphylactic type of reaction mediated through serum and characterized by wheal and flare, there is an immediate increase in vascular permeability (McMaster, 1959). It was therefore decided to transfer serum from mice having past experience of subcutaneous staphylococcal infection with strain PS80 to new mice and to compare their response to the subsequent injection of 10^5 *Staph. aureus* strain PS80 mixed with cotton-dust with that of normal mice.

Lesion score.—Only mild lesions were produced. The average lesion score based on 2 experiments, of 6 mice each was 3·0. The reduction in the severity of the lesion was due to neutralization of dermonecrotic effect.

Fluid exudation.—All the 4 mice that received serum showed some exudation of fluid at ½ hr. (Table III). In contrast all the control mice showed little or no exudation at this stage. At 24 hr. the serum-treated group had more oedema fluid in the lesion than the control group. Although the numbers are small, the range of mean oedema weight shown by the individual mice suggests that the differences between serum treated and control mice may well be real.

TABLE III.—*Fluid Exudation in Serum Treated and Untreated Mice After the Injection of* Staph. aureus *PS*80 *and Cotton*

Time after injection of cocci (hr.)	Weight of oedema fluid (mg.)	
	Serum treated group	Control group
½	5·7 (0·2–19·3)*	−6·0 (−16·7–3·9)
24	154·8 (120·9–187·2)	111·7 (14·2–168·9)

* = In brackets is the range of mean oedema weight.

Leucocyte emigration.—In the serum-treated mice emigration of leucocytes was detected at 2 hr. and the cotton itself showed some evidence of infiltration even at this time. This is in marked contrast to control mice in which no infiltration of cotton was seen until 7 hr. after inoculation, although leucocyte emigration was evident at 2 hr.

Bacterial count.—The counts of viable cocci in the lesions of serum-treated and control mice are shown in Fig. 6. The counts in the lesions of the 2 groups were similar. The growth rate of the cocci was the same in both groups (Table IV).

TABLE IV.—*Bacterial Counts in the Lesion of Serum-Treated and Control Mice*

Group	Initial	"Zero-hr." count $\times 10^3$	Time (hr.) of lowest count	Count at 2 hr. $\times 10^2$	Growth rate k./hr.	Counts at 7 hr. $\times 10^4$	Counts at 24 hr. $\times 10^6$
Serum treated	5·5	3·13	2	3·4	0·45	9·1	2·6
Control	3·81	2·83	2	9·3	0·40	18·3	16·8

Lymph-node cells transfer

Lymph node cells from mice having past experience of subcutaneous staphylococcal infection with the strain PS80 were transferred to new mice. In these

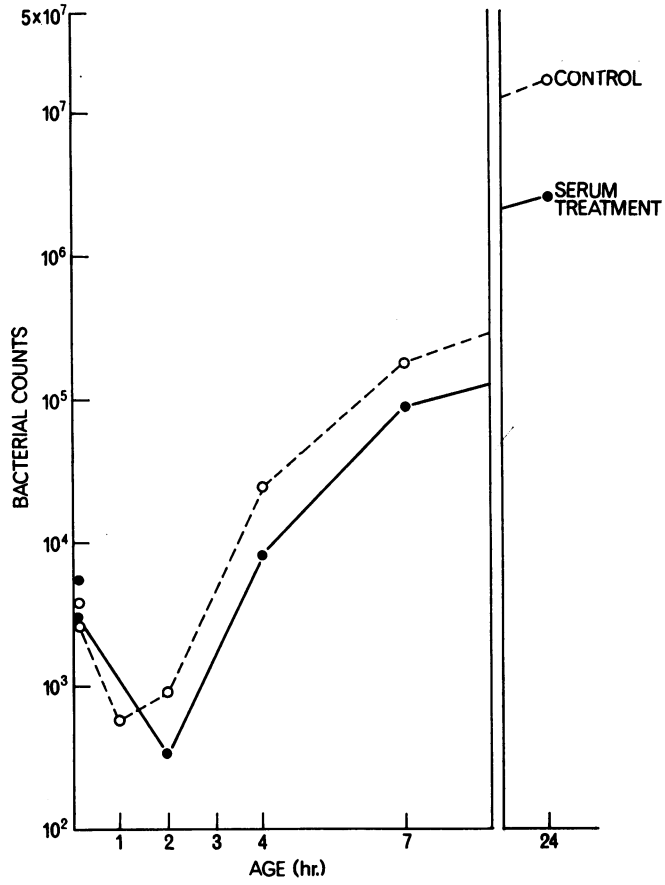

FIG. 6.—Bacterial counts in the lesion after the s.c. injection of *Staph. aureus* strain PS80 mixed with cotton-dust in serum treated and control mice. Each point on the graph is the mean value from 4 mice.

experiments only small numbers of mice were used so that the conclusions may not have the same validity as those from earlier experiments.

The treated mice showed very severe lesions, which were characterized by large areas of necrosis. The average diameter of the necrotic zone after an injection of 10^4 staphylococci was 6·2 mm. and the average lesion score was 4·4. Six control mice injected at the same time with the same dose had on average a necrotic zone 2·2 mm. in diameter and a lesion score of 3·6.

Fluid exudation and leucocyte count were estimated only at 24 hr. after the inoculation of cocci. The mice receiving lymph node cells showed less fluid exudation at 24 hr. than the control mice (Table V).

In the histological sections of the lesion at 24 hr., both with the 10^4 and the 10^5 dose of staphylococci, there was only slight infiltration of the cotton-dust by leucocytes. Only the peripheral part of cotton was infiltrated and in places the leucocytes even remained away from its edge. In contrast, in the untreated mice,

TABLE V.—*Fluid Exudation in Mice given Lymph Node Cells and Control Mice 24 hr. After the Injection of* Staph. aureus *PS80 Mixed with Cotton-Dust*

Experiment No.	Dose of PS80 $\times 10^5$	Lymph node cells treated mice: Oedema weight (mg.)	Lymph node cells treated mice: No. mice	Control mice: Oedema weight (mg.)	Control mice: No. mice
1	2·5	67·3 (35·7–115·3)*	4	85·3 (73·8–114·0)	4
2	0·2	55·1 (−0·4–99·6)	4	97·1 (59·7–118·3)	4

* = Range of mean oedema weight.

with both the 10^4 and 10^5 dose of PS80, there was a heavy infiltration of the entire cotton-dust by leucocytes at 24 hr.

Bacterial counts were made of lesions at 7 and 24 hr. but showed no difference.

Transfer of lymph-node cells and serum

Ten mice were each given $1·2 \times 10^8$ lymph-node cells and 0·25 ml. of serum by the intravenous route. Eighteen hours later these mice, together with a group of untreated mice, were injected with a 10^5 dose of *Staph. aureus* PS80 in cotton-dust (Table VI). The lesions produced in the treated mice were mild; with an average

TABLE VI.—*Response to Injection of* Staph. aureus *PS80 and Cotton in Mice Treated with Serum + Lymph Node Cells*

Group	Dose of PS80 $\times 10^5$	Average lesion score	Mean oedema weight at 24 hr. (mg.)	Infiltration of cotton by leucocytes at 24 hr.
Control	7·75	4·4	128·5	++++
Treated with lymph node cells + serum	7·75	2·5	127·1	++++

The lesion score is an average from 6 mice each. The value of oedema weight in each group is the mean from 4 mice.

lesion score of 2·5 compared to 4·4 in the control mice. There was no evidence of an increased exudation of fluid in the 24 hr.-old lesions of treated mice. As already described, PI mice had shown greater exudation of fluid at 24 hr. than the controls. There was a heavy infiltration of the cotton-dust by leucocytes at 24 hr., as in the control mice.

The effect of anti-inflammatory drugs on the course of staphylococcal infection in mice

In almost all the studies of the effect of anti-inflammatory drugs, the degree of fluid exudation and leucocyte emigration were estimated at only one time, namely 4 hr. after inoculation. The response at 4 hr. was chosen for study, because of the indications that, in experimental bacterial infections, most of the reactions determining the ultimate size of the local lesion are complete in 4–5 hr. (Miles, Miles and Burke, 1957). In all experiments untreated mice were included as controls. The various anti-inflammatory drugs were tested in separate experiments. The studies reported here were exploratory and would require considerably more work to enable firm conclusions to be drawn.

Fluid exudation.—The oedema weight in the lesions of drug-treated mice was compared with the oedema weight in controls (Table VII). As in each group only

TABLE VII.—*Mean Oedema-Weight of Tissue in the Drug-Treated Mice 4 hr. After the Injection of* 10^5 Staph. aureus *Strain PS80 Mixed with Cotton-Dust*

Exp. No.	Treatment	No. mice	Mean oedema weight (mg.)	Mean blue diameter (mm.)
1	Control	5	14·08 (−11·4–24·6)*	9·3 (7·3–10·8)*
	Cortisone acetate	6	11·3 (−25·9–44·4)	9·4 (6·9–11·4)
	Betamethasone disodium phosphate	5	− 0·4 (−30·7–15·4)	7·1 (2·8– 9·7)
	Dexamethasone-21-phosphate disodium salt	4	−12·4 (− 1·6––37·6)	8·7 (6·0–11·8)
2	Control	5	64·8 (9·3–140·1)	11·8 (9·9–14·0)
	Butazolidin	5	7·4 (−13·1–52·2)	7·9 (6·0–11·9)
	Prednisolone disodium phosphate	5	8·3 (1·5–43·2)	8·5 (5·2–11·4)
3	Control	4	65·3 (42·3–79·0)	13·7 (10·9–15·8)
	Colchicine	4	18·4 (−17·3–50·7)	7·7 (5·0–11·0)
4	Control	6	41·8 (13·0–66·4)	13·3 (11·4–15·0)
	Chlorpromazine hydrochloride	5	39·3 (6·3–79·3)	12·6 (8·9–17·3)
	Quinine hydrochloride	5	46·7 (31·8–65·7)	15·5 (10·9–18·9)
	Colchicine	5	10·3 (−1·4–32·3)	8·7 (7·7– 9·7)
5	Control	4	18·5 (16·0–20·2)	—
	Indomethacin	4	25·6 (2·9–60·6)	—

* = In brackets the range of mean oedema weight and mean blue diameter in the mice tested is given.
— = Not tested.

small numbers of animals were used and the mice within any group often showed considerable variation, very few differences between the drug-treated and control mice were significant. In Table VIII the oedema weights in the drug-treated mice are expressed as a ratio to the oedema weight in the controls. A ratio of 0·5 or less 4 hr. after the injection of staphylococci was arbitrarily regarded as indicative of a reduction in the fluid exudation. Betamethasone, dexamethasone, butazolidin, prednisolone and colchicine gave oedema weights which were 0·5 or less of the controls; chlorpromazine, quinine and indomethacin failed to suppress the fluid exudation.

TABLE VIII.—*Oedema Weight of the Tissue in Drug-Treated Mice 4 hr. After the Injection of* 10^5 Staph. aureus *Strain PS80 Mixed with Cotton-Dust, expressed as Ratio to Oedema Weight in Controls*

Exp. No.	Treatment	Oedema weight as ratio to weight in controls
1	Cortisone acetate	0·81
	Betamethasone disodium phosphate	<0·1
	Dexamethasone-21-phosphate disodium salt	<0·1
2	Butazolidin	0·12
	Prednisolone disodium phosphate	0·13
3	Colchicine	0·29
4	Chlorpromazine hydrochloride	0·94
	Quinine hydrochloride	1·1
	Colchicine	0·25
5	Indomethacin	1·3

Leucocyte count.—The results of leucocyte counts are shown in Table IX. Only colchicine, prednisolone, dexamethasone, betamethasone and cortisone reduced the leucocyte count to ½ or less of that in the controls.

TABLE IX.—*Total Leucocyte Count per HPF in the Tissue Section of Drug-Treated Mice 4 hr. After the Injection of* Staph. aureus *Strain PS80*

Exp. No.	Treatment	Total leucocytes per H.P.F.	Leucocyte count in drug-treated mice as ratio of counts in controls
1	Control	319	—
	Colchicine	104	0·33
	Chlorpromazine hydrochloride	458	1·43
	Quinine hydrochloride	518	1·62
	Butazolidin	461	1·44
2	Control	456	—
	Prednisolone disodium phosphate	208	0·46
3	Control	227	—
	Indomethacin	297	1·3
4	Control	517	—
	Dexamethasone-21-phosphate disodium salt	122	0·24
	Betamethasone disodium phosphate	196	0·38
	Cortisone acetate	237	0·46

Each value represents the mean from the histological preparations of 2 mice and in each tissue section the counts were done in 3 to 4 fields.

Lesion score.—The severity of the staphylococcal lesion after the administration of various drugs was evaluated 24 and 48 hr. after the injection of cocci (Table X). In all cases the higher lesion score was due to a bigger zone of necrosis in the test mice than in the control mice. Another estimate of the lesion enhancement was therefore obtained by measuring the mean diameter of necrotic lesion. The mean diameter of necrosis in the drug-treated mice is also expressed as a ratio to that in the controls (Table X).

TABLE X.—*Staphylococcal Lesions in Mice After the Administration of Anti-Inflammatory Drugs*

Exp. No.	Treatment	No. of mice	Average lesion score	Mean diameter of necrosis (mm.)	Mean diameter of necrosis in treated mice as ratio to diameter in controls
1	Control	6	4·8	4·2	—
	Quinine hydrochloride	6	4·0	2·8	0·7
	Chlorpromazine hydrochloride	6	4·3	3·1	0·7
	Betamethasone disodium phosphate	6	4·6	5·7	1·4
	Dexamethasone-21-phosphate disodium salt	6	5·3	6·2	1·5
	Butazolidin	6	5·3	8·7	2·1
	Cortisone acetate	6	5·1	8·9	2·1
	Prednisolone disodium phosphate	6	5·6	9·5	2·3
2	Control	6	3·5	2·2	—
	Colchicine	6	5·8	9·8	4·4
3	Control	6	3·8	2·8	—
	Indomethacin	6	4·5	4·5	1·6

Apart from quinine and chlorpromazine hydrochloride, all the drugs tested enhanced the severity of the lesions to some extent. However, only butazolidine, cortisone, prednisolone and colchicine produced lesions double the size of the controls. It is noteworthy that of the 4 corticosteroids tested, betamethasone and dexamethasone reduced the fluid exudation to a greater extent than cortisone and prednisolone and reduced leucocyte emigration to about the same extent, yet the staphylococcal lesions in both dexamethasone- or betamethasone-treated mice were smaller than in the mice treated with cortisone or prednisolone. It may be that the enhancement of staphylococcal lesions by corticosteroids is not solely due to suppression of inflammation.

Relation of the 2 components of inflammation to lesion severity.—The effects of the anti-inflammatory drugs are summarized in Table XI. Of the drugs tested

TABLE XI.—*The Effect of Anti-Inflammatory Drugs on Staphylococcal Infection*

Drug	Reduction in fluid exudation	Reduction in leucocyte emigration	Enhancement of lesion
Quinine hydrochloride	−	−	−
Chlorpromazine hydrochloride	−	−	−
Indomethacin	−	−	−
Cortisone acetate	−	+	+
Prednisolone disodium phosphate	+	+	+
Betamethasone disodium phosphate	+	+	−
Dexamethasone-21-phosphate disodium salt	+	+	−
Colchicine	+	+	+
Butazolidin	+	−	+

quinine, chlorpromazine and indomethacin affected neither component of inflammation at 4 hr. and had no effect on the severity of the lesion. Of the 4 corticosteroids tested, cortisone reduced leucocyte emigration without reducing the fluid exudation, while betamethasone, dexamethasone and prednisolone reduced both the components of inflammation; only cortisone and prednisolone were able to produce lesions twice or more as big as the controls. Colchicine reduced both the components of inflammation and also enhanced the lesion. Butazolidin reduced fluid exudation without affecting leucocyte emigration and increased the lesion severity.

The action of cortisone acetate in enhancing the severity of the lesion seemed to be dependent on the number of staphylococci injected. With a dose of 10^2–10^3 cocci, cortisone treatment either failed to enhance the lesion severity or even reduced the lesion size compared with control mice (Table XII). With 10^4–10^5 staphylococci the cortisone-treated mice showed large areas of necrosis and a higher lesion score than the controls. With the 10^3 dose, the average lesion score in the cortisone-treated animals was affected substantially by 2 mice out of 6 tested, which had severe lesions. With the lowest dose (10^2) the lesion score in the cortisone-treated mice was much lower than in the controls.

Bacterial counts.—Bacterial counts in the lesions were done only in cortisone, butazolidine and colchicine-treated mice. The results are shown in Table XIII, which also shows the bacterial counts in the lesions of untreated control mice. As the action of cortisone in enhancing the lesion severity was found to be depen-

TABLE XII.—*The Relation of Lesion Severity in Cortisone-Treated Mice to the Dose of* Staph. aureus *Strain PS*80

Dose of PS80	Cortisone treated mice: No. of mice	Cortisone treated mice: Average lesion score	Cortisone treated mice: Mean diameter of necrosis (mm.)	Control mice: No. of mice	Control mice: Average lesion score	Control mice: Mean diameter of necrosis (mm.)
$6{\cdot}0\times10^{5}$	6	5·1	8·8	6·0	4·8	4·2
$5{\cdot}6\times10^{4}$	6	5·3	9·1	6·0	3·6	4·2
$5{\cdot}66\times10^{3}$	6	2·8	*	6·0	2·5	†
$9{\cdot}0\times10^{2}$	6	0·5	Nil	6·0	2·5	<1·0

* Of the 6 mice tested only 2 showed necrosis of 4·6 mm. and 12·8 mm. in diameter respectively.
† Of the 6 mice tested only one showed a necrotic zone of 2 mm. in diameter.

TABLE XIII.—*Bacterial Counts in the Lesions of Drug-Treated and Control Mice After the Injection of* Staph. aureus *PS*80 *Mixed with Cotton-Dust*

Treatment	Initial dose $\times10^{5}$	"Zero-hr." count $\times10^{5}$	2 hr. count $\times10^{5}$	Rate of growth k./hr.	Counts at 7 hr. $\times10^{5}$	Counts at 24 hr. $\times10^{5}$
No drug (control)	4·0	Not tested	0·81	0·51	241·0	591·0
	0·3	0·16	0·046	0·40	5·6	195·0
	0·038	0·028	0·009	0·40	1·83	168·0
Cortisone	2·5	0·39	0·39	0·53	198·0	1410·0
	0·53	0·085	0·23	0·41	28·6	260·0
	0·056	0·013	0·006	0·29	0·17	749·0
Colchicine	0·06	0·027	0·002	0·40	0·25	309·0
Butazolidin	0·07	0·019	0·003	0·40	0·34	308·0

Each value is the mean for 2 mice.

dent on the number of staphylococci injected, bacterial counts in cortisone-treated mice were done after injecting varying numbers of staphylococci. With colchicine and butazolidin the bacterial counts of the lesion were done only with an original dose of 10^{3} *Staph. aureus* strain PS80.

In all cases the bacterial counts in the drug-treated mice were similar to those in the lesions of control mice, except with the 10^{3} dose in cortisone-treated mice, in which the rate of growth of cocci per hr. (*k.*/hr.) was 0·29 as compared with 0·40 in the controls. At 24 hr. the bacterial counts were very similar in all cases irrespective of treatment or dose.

The effect of endotoxin on staphylococcal infection

Mice were first injected with endotoxin and immediately afterwards were challenged by the s.c. injection of 10^{5} *Staph. aureus* strain PS80 mixed with cotton-dust.

Lesion score.—In endotoxin-treated mice, very severe lesions were produced after the injection of staphylococci. The average lesion score from 16 mice in 3 different experiments was 5·7, compared with 4·8 for the controls. The lesions were mainly necrotic. The mean diameter of the necrotic zone was 11·6, *i.e.* nearly 3 times the 4·2 mm. zone of the controls.

Fluid exudation.—There was very little fluid exudation up to the first 3 hr. of challenge (Fig. 7). Some exudation of fluid started after 3 hr., but was slight even up to 7 hr. In contrast, fluid exudation was quite marked after 2 hr. in the untreated mice.

Leucocyte emigration.—The leucocyte response did not start until about 4 hr. after the injection of cocci. Although the cotton itself showed evidence of slight infiltration at 4 hr., it was not complete even at 24 hr. when only a small part of the periphery of the cotton-dust was infiltrated.

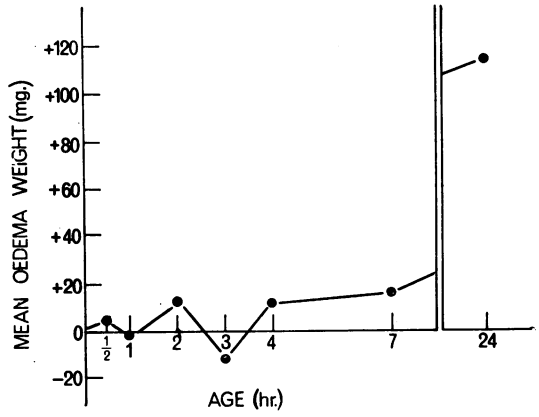

FIG. 7.—The mean oedema weight of tissue after the s.c. injection of 10^5 *Staph. aureus* PS80 mixed with cotton-dust in endotoxin treated mice. Each point on the graph is the mean value from 4 mice.

Possible mechanism of action of endotoxin in enhancing the severity of staphylococcal lesions.—Besides affecting the inflammatory response, endotoxin might enhance the staphylococcal lesions in other ways. In view of the large areas of necrosis produced by staphylococcal injection, it seemed possible that endotoxin might be enhancing the action of α-haemolysin.

" Wellcome " brand α-haemolysin (0·16 u.) was injected with cotton-dust s.c. into mice that had been treated with endotoxin. As a control α-haemolysin was also injected into untreated mice. For comparison mice treated with cortisone acetate and colchicine were also included in the experiment. In none of the treated mice was the zone of necrosis twice as big as in the controls (Table XIV),

TABLE XIV.—*The Effect of Endotoxin and Anti-Inflammatory Drugs on the Dermonecrosis Produced by α-Haemolysin*

Treatment	No. of mice	Average diameter of necrosis (mm.)	Diameter of necrosis as ratio of diameter of necrosis in control
None (control)	6	3·8 (2·4–4·6)*	—
Cortisone acetate	6	4·5 (2·0–7·3)	1·2
Colchicine	6	3·3 (2·5–7·2)	0·87
Endotoxin	6	5·2 (4·4–6·6)	1·3

* The range of diameter of necrosis is given in brackets.

so the enormously big areas of necrosis produced after injection of staphylococci in endotoxin-treated mice seemed not to be due to enhancement of the dermonecrotic action of α-haemolysin.

In view of the similarity between the interaction of staphylococci and endotoxin and the effect of adrenalin upon endotoxin-prepared skin of rabbits (Thomas, 1959), an experiment was done using the adrenergic blocking agent " Dibenyline ". Twelve mice were given " Dibenyline " (Smith, Kline and French) i.p. in 2 doses of 50 μg. at 24 and 2 hr. before the injection of endotoxin or colchicine. Colchicine was included in the experiment to see whether its infection-enhancing activity could also be blocked by Dibenyline. All the 24 mice were injected s.c. with 10^5 *Staph. aureus* strain PS80 mixed with cotton dust. The severity of the lesion is expressed in terms of mean diameter of necrotic zone in Table XV. There was

TABLE XV.—*The Effect of Dibenyline on the Staphylococcal Lesion of Endotoxin- and Colchicine-Treated Mice*

	Mean diameter of necrosis (mm.)	
Group	Endotoxin treatment	Colchicine treatment
Control	14·3 (6·3–21·1)*	9·3 (5·0–17·4)
Dibenyline	8·5 (5·0–12·5)	7·2 (4·6–13·5)

* The range of diameter of necrosis is given in brackets.

some reduction in the severity of staphylococcal lesion in the endotoxin-treated mice after Dibenyline administration: the diameter of necrosis was 59 per cent of that in the controls, as compared with 78 per cent of the controls in colchicine-treated mice. Thus Dibenyline did not completely abolish the infection-enhancing activity of endotoxin; even after its administration, the lesions in endotoxin-prepared mice were twice as big as in the untreated mice injected with a comparable dose of cocci.

DISCUSSION

Mice having past experience of subcutaneous staphylococcal infection were protected against the dermonecrotic effects of staphylococci and of α-haemolysin. When challenged with the homologous staphylococcus in cotton-dust, the lesion that developed was a subcutaneous abscess with no overlying necrosis, like that produced by less virulent staphylococci (Agarwal, 1967*b*). The protection against dermonecrosis was mediated by serum and was presumably due to the presence of antibodies to α-haemolysin.

These mice also exhibited an accelerated inflammatory response to a new infection with staphylococci; with a 10^3 dose of *Staph. aureus* PS80 there was a slower rate of growth of cocci in the lesions than in normals and 24 hr. bacterial counts were lower than the dose inoculated.

The accelerated increase in vascular permeability might be due to a hypersensitivity reaction to staphylococci. Both the immediate as well as the delayed type of hypersensitivity reaction to staphylococci and their products have been reported (Julianelle and Wieghard, 1935; Julianelle and Hartmann, 1936; Johnson *et al.*, 1961; Goshi, Smith, Cluff and Norman, 1963; Bodel and Atkins, 1964). Moreover Voisin and Toullet (1960) have reported an increase in vascular

permeability in both the immediate as well as the tuberculin type of hypersensitivity. In the present study serum from " previously-infected " mice was able to confer some acceleration of the inflammatory reaction on normal mice. This suggests that the accelerated exudation of fluid and the rapid leucocyte emigration as well as the infiltration of the cotton-dust containing the staphylococci was due to an immunological response mediated by circulating antibodies. The rapid emigration of leucocytes could be due to a chemotactic effect exerted by antigen-antibody complex, which is claimed to have a strong chemotactic effect on polymorphonuclear leucocytes (Boyden, 1962). On the other hand the rapid leucocyte infiltration of the cotton-dust might well be due to neutralization of staphylococcal α-toxin, because in " previously-infected " mice the leucocytes rapidly infiltrated the cotton-dust containing α-haemolysin. Goshi, Cluff and Norman (1963) have also demonstrated prompt infiltration of the area of staphylococcal inoculum by polymorphonuclear leucocytes in the animals immunized with α-haemolysin toxoid, in contrast to normals.

The nature of the antigen or antigens responsible for sensitization can only be surmised. This work has illustrated that both the α-haemolysin and some component associated with the staphylococcal cells are involved. In previously-infected mice there was an acceleration in the exudation of fluid in response to both killed staphylococci and α-haemolysin; this is in agreement with the findings of Goshi *et al.* (1963). Other staphylococcal antigens incriminated in the hypersensitivity reaction include polysaccharide A for the immediate type of reaction (Julianelle and Hartmann, 1936) and a protein antigen common to both the pathogenic and non-pathogenic strains of staphylococci for the delayed reaction (Julianelle and Wieghard, 1935).

In this work repeated staphylococcal infection produced no increased susceptibility as might be expected in a delayed hypersensitivity reaction. This is in contrast to the findings of other workers (Johnson *et al.*, 1961; Johanovský, 1958). However, when living lymph-node cells from " previously-infected " mice were transferred to normal mice, the treated mice exhibited a markedly increased susceptibility. On the other hand when lymph-node cells were transferred along with the serum from " previously-infected " mice, there was no increase in susceptibility to staphylococci in the treated mice; in fact, they exhibited protection against dermonecrosis. This suggests that, in the presence of antibodies to products of staphylococci, the increased susceptibility mediated by lymph-node cells is not manifested.

The importance of the early inflammatory response in limiting the staphylococcal infection has been reported (Agarwal, 1967*a*, *b*). It seems that the accelerated inflammatory response of the " previously-infected " mice led to the low bacterial counts in the lesion, presumably because of the destruction of cocci.

The suppression of the inflammatory reaction by the use of anti-inflammatory drugs also altered the staphylococcal lesion. Drugs that reduced one or both components of staphylococcal inflammation, *e.g.* colchicine, butazolidine, cortisone acetate and prednisolone, were found to enhance the severity of the lesions. However, betamethasone and dexamethasone, in spite of their anti-inflammatory activity, did not increase the lesion severity in the dosage used. Drugs like quinine hydrochloride, chlorpromazine hydrochloride and indomethacin, which did not alter the inflammatory response during the first 4 hr., failed to increase the susceptibility of the mice to staphylococci.

The role of inflammatory cells as the major agent in the destruction of staphylococci is well established (Cohn, 1962*a*, *b*); the role of the fluid part of the exudate in the response to staphylococcal infection is not certain. The results of treating mice with anti-inflammatory drugs suggest that the fluid part of the exudate also plays an important role. For example, treatment of mice with butazolidin, which was found to reduce the fluid exudation without affecting leucocyte emigration, enhanced the severity of staphylococcal lesions. However, it is not known whether butazolidin affects phagocytosis or eventual destruction of cocci inside the cell. The " infection-enhancing " effect of most of the anti-inflammatory drugs may be due to their action on host defence mechanisms at several levels.

The action of corticosteroids in enhancing the staphylococcal infection was associated with decreased leucocyte emigration, which presumably affected the destruction of cocci; however this destruction could not be substantiated by the method of bacterial counting used in this study. Another complex aspect of cortisone action was that, with low doses of cocci, the lesions were milder in cortisone-treated than in normal mice. This may be due to the stabilizing effect of cortisone on lysosomes (Weissmann and Thomas, 1964) and perhaps, in spite of the reduced inflammatory response, the tissue defences were adequate to deal with the small numbers of cocci injected. As already suggested, there may be some other mechanism reducing the host resistance beside the anti-inflammatory activity of these drugs.

The infection-enhancing activity of endotoxin has been attributed to a delay in the emigration of leucocytes (Miles and Niven, 1950; Conti, Cluff and Scheder, 1961; Cohn, 1962*b*). The findings of this work illustrated that endotoxin treatment reduced both the fluid exudation and the leucocyte emigration and allowed the staphylococci to produce big necrotic lesions. There was no evidence to suggest that the large necrotic areas in the endotoxin-treated mice were due to increased susceptibility of these mice to α-haemolysin. Besides the anti-inflammatory activity other actions of endotoxin may also be involved in enhancing the infection. Administration of the adrenergic blocking agent Dibenyline in endotoxin treated mice reduced the severity of staphylococcal lesions, a finding similar to that reported by Sultzer and Freedman (1965). However, the enhancing activity of endotoxin was not completely abolished and the lesions were still twice as big as in the untreated mice.

SUMMARY

Mice having past experience of subcutaneous staphylococcal infection were protected against the dermonecrotic effects of staphylococci and of α-haemolysin. After challenge with homologous staphylococci in cotton-dust, these mice exhibited an early exudation of fluid evident $\frac{1}{2}$ hr. after the injection, in contrast to normal mice, in which it started after an initial delay of 2 hr. At 24 hr. there was much more oedema fluid in the lesions of " previously-infected " mice than in control mice. The emigration of leucocytes started 1 hr. after injection in contrast to 2 hr. after injection in normal mice and the cotton-dust which contained the cocci was infiltrated 3–4 hr. after injection, *i.e.* much earlier than in the normal mice. With a 10^3 dose of *Staph. aureus* strain PS80, there was a slower rate of growth of cocci in the lesions of " previously-infected " mice than in normals and 24-hr. bacterial counts were lower than the dose inoculated.

Passive transfer of serum from " previously-infected " to normal mice protected

them from the dermonecrotic effects of staphylococci and the treated mice also showed accelerated exudation of fluid and rapid emigration of leucocytes as well as the infiltration of the cotton-dust by them.

Injection of heat-killed staphylococci of the homologous strain, as well as α-haemolysin, produced an earlier exudation of fluid in the " previously-infected " mice than in normals.

Transfer of lymph-node cells from " previously-infected " to normal mice led to an increased susceptibility of the treated mice to the s.c. injection of staphylococci.

Amongst the anti-inflammatory agents tested, colchicine, butazolidin, cortisone acetate, prednisolone and endotoxin enhanced the severity of staphylococcal lesions and reduced one or both components of staphylococcal inflammation. However, some anti-inflammatory agents like betamethasone and dexamethasone, in spite of their anti-inflammatory activity did not increase the lesion severity in the dosage used.

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