THE PRODUCTION OF SUBCUTANEOUS STAPHYLOCOCCAL SKIN LESIONS IN MICE*

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MANY attempts have been made to find a laboratory test that would indicate the " virulence " or " infectivity " of staphylococci for man. In assessing the adequacy of any such test we must consider whether the test is realistic in relation to human staphylococcal disease and whether the results of the tests are correlated with the virulence of the staphylococci for man. In man, the number of deaths due to staphylococcal infection is small compared with the amount of disease (Public Health Laboratory Service, 1960) and it would seem, therefore, that an animal test leading to the production of non-fatal disease would be more likely to give a result which had some relevance for man, than would techniques leading to death of the animal.

Very large doses of staphylococci are needed, in both man and animals, to cause skin infection by simple injection, but implantation of the staphylococci on cotton sutures has been found markedly to reduce the dose needed (Elek and Conen, 1957 ; James and MacLeod, 1961). Since it seems unlikely that, in the production of natural staphylococcal disease, more than a few staphylococci are introduced initially to the site at which the lesion is to occur, potentiation of infection by sutures or crushed tissue may be of considerable importance. The suture appears to act as a mechanical depot for the staphylococci and also perhaps as an irritant.

The use of sutures tied through the skin to produce lesions in experimental animals has, however, two major disadvantages ; many of the sutures may be bitten or scratched out by the animal, unless the area is covered, or the ends of the suture sealed to the skin ; and, in addition, the dose of bacteria actually administered may be difficult to assess.

Cotton dust had proved a satisfactory substrate on which to study the survival of staphylococci during drying (Noble, 1961) because a suspension of the cotton in saline or broth could be pipetted as though there were no solid phase present. It was thought therefore that subcutaneous introduction of infected cotton dust might be an improvement on the introduction of cotton sutures, for there would be no risk of its being removed by the animal. Similar methods have been used to study the formation of granulation tissue in rats (Penn and Ashford, 1963).

A method for the production of subcutaneous lesions in mice by the subcutaneous introduction of staphylococci on plugs of cotton dust was therefore developed and is described here. The relation of the severity of the lesions

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produced to the infectivity of staphylococci for man and to the biochemical characteristics of the staphylococci will be described separately. A full discussion of published material relating to this work may be found in the thesis which forms the source of this paper.

MATERIALS AND METHODS

Cotton dust

The dust used was C4 white cotton flock, which is pure cellulose and corresponds to the cotton in a bleached cotton fabric. It was obtained from the Shirley Institute, Didsbury, and Messrs. Hutchinson (Ramsey) Ltd., to whom I am grateful.

Staphylococci

Many strains were investigated but 4 were selected as " standards ". These were :

PS 80 (NCTC 9789). The propagating strain for phage 80. This strain was originally isolated by Dr. P. M. Rountree in Sydney ; it is phage-type 80/81 and resistant to penicillin only.

Wood 46 (NCTC 7121).—This strain produces large quantities of α-haemolysin *in vitro* ; it is phage-type 47+ and sensitive to all antibiotics.

Charlton.—Isolated from a severely septic lesion during an outbreak of cross-infection in a surgical ward, phage-type 53/77, and resistant to penicillin, tetracycline and streptomycin.

Type 52.—Isolated from a nasal swab during a survey of healthy carriers, phage-type 52 and sensitive to all antibiotics.

Mice

Mice of the Wright-Fleming Institute strain were used throughout these experiments. There was no significant difference between the reactions given to challenge by male and female mice (10 per cent $> P >$ 5 per cent). A few investigations showed that mice of the I.C.I. strain gave identical results to those obtained in Wright-Fleming Institute mice.

Method

The cotton was introduced under the skin of the mouse with a transfusion needle fitted with a wire plunger to expel the dust (Fig. 1). The plunger was marked so that it could be withdrawn a given distance (0·5 cm.) up the barrel of the needle ; the needle was then filled by packing loose cotton dust into the space in the lumen of the needle between the point and the plunger ; in this way about 2·5 mg. of cotton dust was used for each plug. The needle was charged either with contaminated cotton dust on which bacteria had been dried or with clean cotton dust. When clean dust was used, the inoculum was picked up on a standard loop and discharged into the cotton by capillarity. The number of viable cocci on the cotton plugs was determined either by suspending control plugs similarly inoculated in saline and plating the suspension on agar, or by inoculating a standard loopful of the staphylococcal culture used to seed the cotton directly on agar. The variation in dose between different plugs inoculated in this way proved to be rather large ; when clean cotton plugs were seeded with a broth culture, the variance of a series of 17 counts was about 7 times that expected from a Poisson distribution (mean count = 126·1, variance = 928·5, n = 17) and when similar plugs made from cotton on which staphylococci had been dried were used, the variance was about 15 times the Poisson variance.

The back of each mouse was clipped and depilated using a barium-chloride powder about 1–2 hr. before inoculation. For inoculation, the skin of the back was pinched up between the fingers, the needle inserted at the base of the fold so that the tip was at least 0·5 cm. from the skin puncture and the cotton plug expelled.

RESULTS

Method of scoring lesion severity

The reactions were recorded daily, for lesions were usually produced within 24 hr. The most severe type of lesion seen consisted of an abscess about 3 mm.

in diameter surrounded by a white or necrotic zone about 1 cm. in diameter. These lesions were given a score of " 3 " when the white or necrotic zone was more than 0·5 cm. in diameter and " 2 " when it was less than this. Some of the lesions showed only a " head " of pus with no white or necrotic zone and were given a score of " 1 " ; lesions that consisted only of a little erythema over the plug itself were scored " 0 ". Fig. 2 shows a severe lesion in which the white zone is visible ; the white zone became necrotic after 2–4 days.

Inoculation of sterile cotton dust gave rise to a slight reaction in about 7 days. No white zone was ever seen when sterile cotton, or cotton infected with micrococci, *Escherichia coli* or *Streptococcus pyogenes*, was injected.

The potentiating effect of cotton dust

Broth cultures of staphylococci were injected subcutaneously into mice and the reaction compared with that produced by a similar inoculum given in cotton dust as described above. The lesions produced in the absence of cotton were,

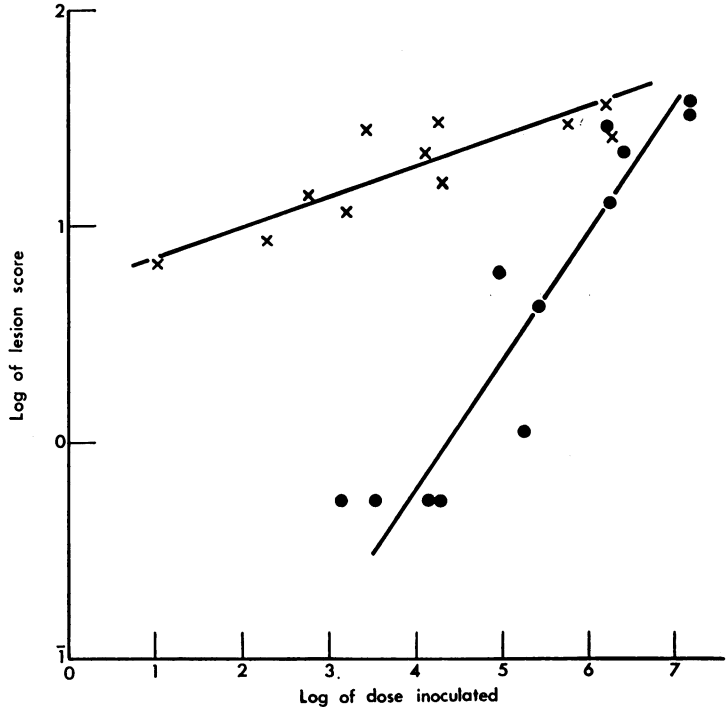


FIG. 3.—Comparison of lesion scores obtained when staphylococcus PS 80 was injected with (×), and without (●) cotton dust.

dose for dose, much less severe than those produced in the presence of cotton dust except at the highest dose levels. The logarithm of the lesion score was plotted against the logarithm of the dose and comparison of the regression coefficients confirmed that the 2 lines were significantly different (Fig. 3, Table I).

TABLE I.—*Comparison of log. dose/log. Response Regression Lines for Staphylococci Injected into Mice With and Without Cotton Dust*

Strain	Regression slope of log. dose against log. score: with cotton	Regression slope of log. dose against log. score: without cotton	*P.* (per cent)	t	Degrees of freedom
PS80	5·8	1·5	<0·1	53·7	21
Type 52	5·3	2·2	<0·1	6·4	14
Wood 46	4·6	1·2	<0·1	18·8	10
Charlton	8·1	1·3	<0·1	40·8	11

The presence of cotton dust thus significantly potentiated the production of subcutaneous lesions by small doses of staphylococci.

Growth of organisms and invasion of the plug by leucocytes

Experiments with staphylococcus PS 80 and staphylococcus Type 52 showed that a very rapid increase in the numbers of organisms in the plug occurred about 4–5 hr. after the plug had been inserted under the skin of the mouse. Fig. 4

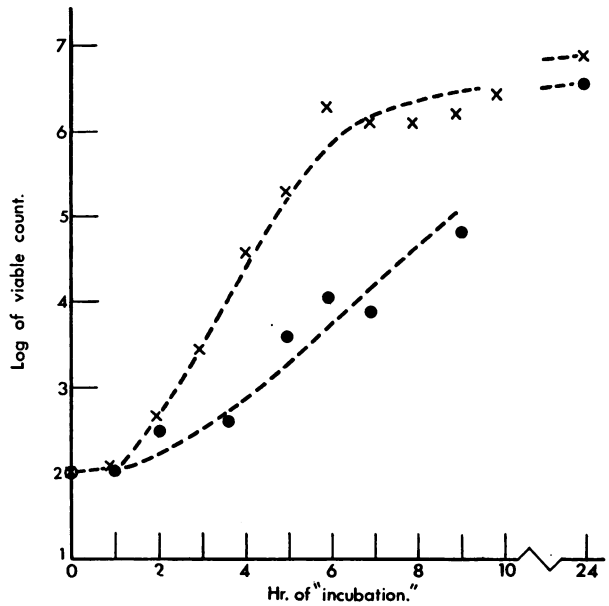


FIG. 4.—Comparison of the growth rates of staphylococcus PS 80 *in vivo* (×) and *in vitro* (●). The actual inoculum subcultured into broth and inoculated into the mouse was of the order of 100 viable units.

shows the growth of staphylococcus PS 80 in broth and under the skin of a mouse, the *in vivo* growth was determined by dissecting out the cotton plugs at intervals after infection and homogenizing them in saline using an M.S.E. homogenizer. At about the same time, the number of leucocytes seen on films made of the plug increased. A number of these leucocytes contained very many staphylococci, for

example 6·5 hr. after inoculation, 26/102 leucocytes appeared to contain cocci and 5 of these contained many cocci. The rather ragged appearance of the leucocytes suggested that the staphylococci were multiplying within the leucocytes rather than that the leucocytes were especially efficient at ingesting cocci.

Histological appearance of the lesions

The histological appearance of a lesion 24 hr. after the insertion of a plug bearing a dose of 10^6 cocci is shown in Figs. 5 and 6. The lesion was a typical abscess ; the cotton plug was infiltrated with leucocytes and thus occupied more space in the tissue than when freshly inserted, and the lesion was walled-off by a thickening of the tissue. The walling-off and infiltration are shown in greater detail in Fig. 6, in which the cotton fibres may be seen.

Dose/response curves for different strains of staphylococci

Dose/response curves for the 4 standard strains were plotted in the form " lesion score " against " logarithm of dose inoculated " and also as " logarithm of lesion score " against " logarithm of dose inoculated ". On statistical grounds there is little to choose between the two transformations, the correlation coefficients being remarkably similar (Table II).

TABLE II.—*Statistical Data for Dose Response Curves*

	Score/log. dose curves		Log. score/log. dose curves		
Strain	Correlation coefficient	Standard deviation* (per cent)	Correlation coefficient	Standard deviation* (per cent)	Degrees of freedom
PS 80	0·96	14·2	0·89	9·3	9
Type 52	0·92	13·8	0·78	13·1	8
Wood 46	0·82	28·6	0·83	11·7	4
Charlton	0·97	7·5	0·92	4·9	5

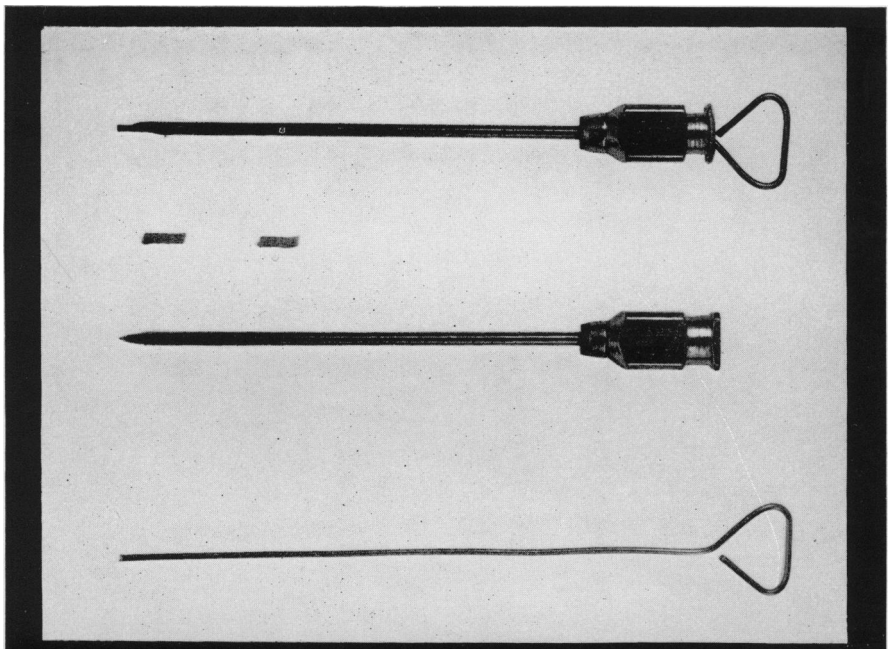
* The standard deviation is given as a percentage of the mean score.

The standard deviations of the score about the regression lines were smaller for the " log. score/log. dose " curves and the regression lines were rather more parallel than those of the other transformation. This transformation was therefore employed and analysis of covariance showed that, when allowance had been made for the differences in dose, there were significant differences in the scores between the four standard strains (5 per cent $> P >$ 1 per cent, $F = 3{\cdot}5$, $n_1 = 3$, $n_2 = 29$).

The standard deviation of the scores about the regression lines " logarithm of score/logarithm of dose " was of the order of 10 per cent (Table II). Provided that a group of 6 or more mice was used for each dose of cocci, the group score obtained by summing the scores for the individual mice was repeatable and reasonably constant for a given dose.

EXPLANATION OF PLATES.

FIG. 1.—Needles and plungers used in test. Two cotton plugs are also shown.
FIG. 2.—Severe lesion. The white and necrotic zone is seen in the centre of the depilated area.
FIG. 5. } Histological appearance of subcutaneous lesions.
FIG. 6. }
FIG. 7.—Micrococcus lesion 10 days after inoculation.



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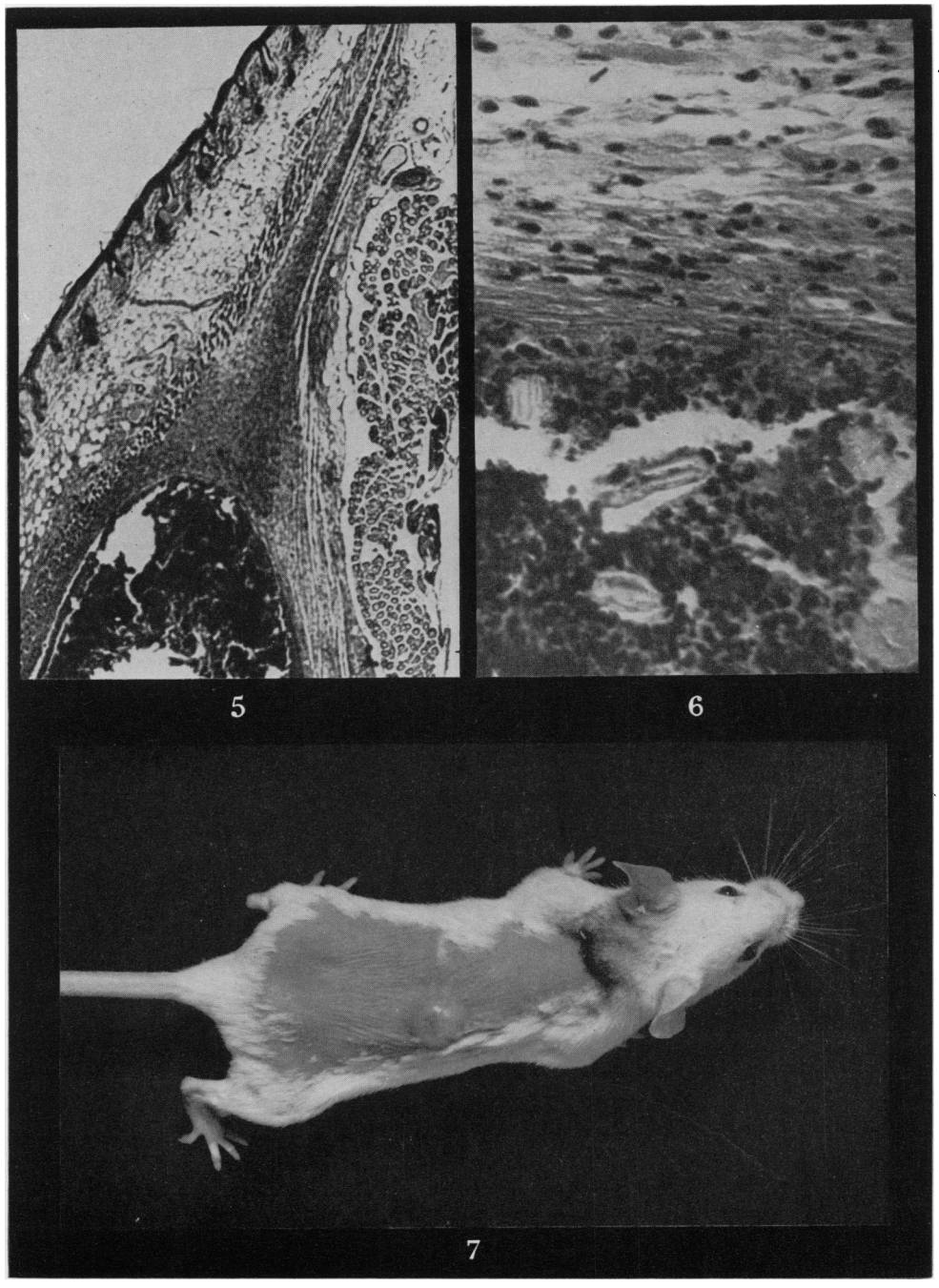

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Life-history of the lesions

Most of the lesions produced by the staphylococci were at a maximum within 1–4 days of inoculation. When a white zone developed in the skin around the lesion it always progressed to necrosis. When necrosis occurred, the cotton plug was discharged and the lesion healed, usually within 10 days, and the fur grew again in about 3–4 weeks.

When a very small dose of staphylococci ($<$ 10 viable units), or a dose of micrococci was introduced, the progress of the lesion was different. Little or no erythema and no external necrosis occurred and the lesion progressed for several days before finally breaking down to release large quantities of pus. Fig. 7 shows a micrococcus lesion 10 days after inoculation.

None of the mice died from generalization of these subcutaneous staphylococcal lesions. Mice were killed at the conclusion of the experiments and 270 were examined *post-mortem*. On no occasion was any micro-organism isolated from the kidneys and on only 3 occasions were staphylococci isolated from the heart blood. There are grounds for believing that these 3 occasions represent faulty necropsy technique. By contrast, many mice infected intraperitoneally or intravenously with staphylococci, or subcutaneously with *Strep. pyogenes*, died of generalized infections and the appropriate organisms were recovered from the liver, spleen, heart and kidneys of all those animals that died.

Investigations into Some of the Factors that Govern Lesion Production

A number of the factors that govern the production of lesions were investigated and are discussed briefly here.

A total of 15 tests on 5 strains of staphylococci showed that washing the cocci in broth-saline prior to inoculation did not significantly alter the severity of the lesions. Hence we may conclude that the severity of the lesions was not dependent on the presence in the inoculum of any preformed toxins.

Adrenalin was not found to have any potentiating effect on the lesions. It seems probable that the cotton dust was already exerting the maximum potentiating effect.

The penetration of material into an infected area was studied by injecting intravenously 0·5 ml. of 0·65 per cent pontamine sky blue. Up to at least 4 hr after infection, the dye penetrated the plug easily, but after 24 hr. the penetration was very slow, the skin around the lesion being deeply stained.

Intramuscular antibiotics in single doses of 5 mg. were effective at preventing the formation of a lesion when given within 4–5 hr. of challenge. Thereafter the antibiotics had little effect on the course of the infection (Table III).

It is thus clear that material present in the blood stream penetrates the lesion in the first few hours after injection of the cotton plug but once the lesion has become established penetration may be slow.

Immunity to infection

Active immunity.—In a joint experiment with Dr. W. D. Brighton of the Applied Immunology Department, The Wright-Fleming Institute, attempts were made to immunize mice against the formation of subcutaneous lesions by vaccination with killed staphylococci of strain PS 80 and with similar vaccines

TABLE III.—*The Effect of Antibiotics on Subcutaneous Infection with Staphylococci*

Strain	Score* Control	Hr. at which 5 mg. antibiotic given 0	3	4	5	6	Total expts. performed
PS80	30·0	4·7	4·9	9·4	25·8	28·6	4
Charlton	32·6	2·6	4·6	6·2	18·6	24·2	2
Wood 46†	14·3	0·4	9·3	7·0	6·0	7·6	3

* Maximum score = 36. 6 mice were used at each point in each experiment and the results given above are the mean score for a number of experiments.

† The apparent effect of antibiotic was less marked in the strains which failed to produce the white zone.

containing denatured toxin (" Vaccoid "). Groups of mice were inoculated intraperitoneally with one dose of vaccine or vaccoid 4 weeks before challenge or with 1 dose 6 weeks, and a further dose 2 weeks before challenge. Four challenge doses of staphylococci were used ranging from 7×10^5 to 7×10^2 cocci of strain PS 80. None of the immunizing schedules appeared to have any effect on the severity of the lesions.

By contrast, it was found that if the mice had experienced a previous subcutaneous staphylococcal infection, the response to a further infection with the same or another staphylococcus was markedly reduced (Table IV). It was noticeable that the white or necrotic zone was rarely seen in these subsequent infections.

TABLE IV.—*The Effect of Repeated Infection with the Same or a Different Staphylococcus*

Initial strain inoculated	Subsequent strain inoculated	Score* Control	1st reinfection	2nd reinfection	3rd reinfection
PS80	PS80	30	14	12	4
Wood 46	Wood 46	26	9	—	—
Charlton	Charlton	30	11	—	—
Type 52	Type 52	11	13	13	—
PS80	Wood 46	34	15	4	—
PS80	Charlton	27	—	9	13
Charlton	PS80	29	10	11	—

* Maximum score = 36. 6 mice were used in each determination.
The results quoted above are those for a challenge dose of 10^6 viable units.

Passive immunity.—Burroughs-Wellcome staphylococcus refined globulins (antitoxin) in doses as low as 25 units of antitoxin per mouse (given intraperitoneally at the same time as the challenge) prevented the formation of the white or necrotic zone without affecting the abscess formation. Some vestiges of the zone were seen when the mice were given 2·5 units of antitoxin. Serum, in doses of 2·5 ml. intraperitoneally, from mice which had experienced 4 previous subcutaneous infections with staphylococcus PS 80, reduced the lesions as much as did 2·5 units of antitoxin.

Injection of 0·07 units of Burroughs-Wellcome staphylococcal α-haemolysin subcutaneously on clean cotton dust produced a white zone in 24 hr. in exactly the same way as did injection of a " virulent " staphylococcus, but failed to produce an abscess. Larger doses produced very severe necrosis within 24 hr. In animals which had had one previous infection, a dose of 0·7 units (10 times the previous dose) was needed to produce the white zone.

Finally it was shown that serum from mice which had experienced 4 previous infections with staphylococcus PS 80, but not that of mice immunized with vaccine or vaccoid, would prevent haemolysis of washed rabbit red cells by a culture supernatant of staphylococcus Wood 46 known to contain α-haemolysin.

All these tests suggest that the white or necrotic zone which surrounds the staphylococcal lesions is produced by α-haemolysin. It may be however that other factors are also involved. As will be described elsewhere, the *in vitro* production of α-lysin by the staphylococci did not exactly parallel the production of the white zone in mice and the severity of the lesions appeared to depend on a number of factors. The Burroughs-Wellcome α-lysin gave 5 antigen/antibody diffusion lines against staphylococcal antitoxin and it is therefore possible that one or more of the other antigens may also have been associated with the appearance of the white or necrotic zone.

DISCUSSION

The subcutaneous injection of staphylococci on plugs of cotton dust gave rise to lesions which to some extent resembled natural human infections, except that the mouse lesions were never accompanied by extensive erythema and human lesions seldom, if ever, exhibit a white or necrotic zone. Mice which had experienced a previous staphylococcal infection produced abscesses but no white zone when reinfected with staphylococci. It has been suggested that man exhibits a sensitivity reaction to staphylococci resembling that produced by injection of pollen antigen in sensitive persons (Smith, Goshi, Norman and Cluff, 1963). Lack of such sensitivity on the part of the mice might explain the failure to produce a marked erythematous reaction.

The advantages of this method are that lesions can be produced regularly and repeatedly with an appropriate dose of staphylococci. None of the mice was able to remove the cotton plug and no dressing was required over the lesion site. Using this technique it is possible to introduce fresh or dry staphylococci into the subcutaneous tissue of the mouse and the dose of cocci inserted, and the standard deviation of that dose, can be easily estimated. Finally the results of the test bear some relation to the infectivity of the staphylococci for man, as will be demonstrated in a subsequent paper.

The experiments reported appear to show that the white or necrotic zone surrounding the staphylococcal lesions was produced by α-haemolysin, although it must be remembered that the Burroughs Wellcome α-lysin contained at least 5 precipitating antigens. It is therefore difficult to be sure that the α-lysin alone was causing the necrosis. Work to be described in a subsequent paper suggests that a number of other factors including β-haemolysin and fibrinolysin may also be of importance in the production of lesions. In studies on the virulence of staphylococci injected intramuscularly into mice (Selbie and Simon, 1952; Howard, 1954), it was found that α-lysin, coagulase, fibrinolysin and β-lysin appeared to play some part in the disease process. Similar conclusions were

reached by Lambert (1960) and Taubler, Kapral and Mudd (1963) following studies on the production of stitch abscesses by infected sutures. The present technique would appear to have the advantage that a definite role, the production of necrosis, can be attributed to one of the products, α-lysin, either alone or in combination with other products of the staphylococcus.

SUMMARY

A new technique for the production of skin lesions in mice is described. The technique depends on the subcutaneous introduction of staphylococci on plugs of cotton dust. The lesions produced tended to resemble human abscesses especially where the animal had previously experienced a staphylococcal skin infection. "Severe" lesions were surrounded by a white or necrotic zone; this zone appeared to be produced by α-haemolysin perhaps in association with other toxins.

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