A STUDY OF VIRULENCE FACTORS WITH INDUCED MUTANTS OF *STAPHYLOCOCCUS AUREUS*

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THE contribution of extracellular products to the virulence of *Staphylococcus aureus* might be investigated by comparing mutants of a strain of known virulence in animal experiments. In studies with experimentally infected sutures in mice, Taubler, Kapral and Mudd (1963) have shown that mutants that had lost the ability to produce α -lysin gave smaller skin lesions than did the wild-type strain. Moreover, the lethal dose for mice of such mutants on intraperitoneal injection was found to be higher than that of the wild type (Kapral, 1965). It thus appears that α -lysin may contribute to virulence for mice. However, the significance of other extracellular products, such as δ -lysin, P-V leucocidin, free coagulase and staphylokinase, in relation to virulence has not been established. In the preceding paper (van der Vijver, van Es-Boon and Michel, 1975), the induction of mutation in a "mouse-virulent" strain is described. In the present paper, the wild-type strain and mutants have been compared in virulence tests that include subcutaneous challenges in normal and irradiated mice, and intravenous challenges in normal mice and in rabbits.

MATERIALS AND METHODS

Strains. The wild-type strain V4, which is virulent for mice, and the mutants have been described in the previous paper (van der Vijver *et al.*, 1975).

Tests for virulence in mice. Mutants and the wild type were compared in the following tests. Normal mice and mice whose general resistance had been reduced by total body X-irradiation were challenged subcutaneously as described elsewhere (van der Vijver, van Es-Boon and Michel, 1974). The severity of the lesions was scored as follows: 0 = no lesion, i.e. absence of pus, infiltration and necrosis, though slight erythema was sometimes present; 1 = presence of pus, infiltration, and a necrotic area or white zone with a diameter less than 5 mm; 2 = necrotic area or white zone with a diameter larger than 5 mm. Viable counts of the organisms in the skin of the mice were determined by cutting out skin patches measuring 2 × 2 cm and disintegrating for 60 s at 0°C in 5 ml of saline according to the method of Fisher and Robson (1963). After disintegration, the collagen fibres were removed by filtration through surgical dressing gauze.

Mice were challenged intravenously by the injection of the stated dose in 0.5 ml of saline into a tail vein. Mortality and abscess formation were scored. Viable counts in the two kidneys taken together were determined after disintegration of the tissue for 5 s.

Bacterial counts in colony-forming units (c.f.u.) were made according to the method of Miles, Misra and Irwin (1938).

Similar experiments were performed in intravenously challenged rabbits.

Received 5 July 1974; accepted 16 Aug. 1974.

RESULTS

Subcutaneous challenge in normal mice

Groups of mice were challenged subcutaneously with various doses of the wild type and mutants given by injection, and the lesion scores were determined after 24 and 48 h. The difference in lesion score after 24 and 48 h was negligible. The lesion scores after 24 h are given in table I and show that all α -lysin mutants gave significantly lower lesion scores than did comparable doses of the wild type. This was due to the absence of necrosis. Mutant BS36 (δ -*lys*⁻, *leuc*^l) differed from the wild type in this respect only at lower doses. Other mutants such as BF34 (*kin*⁻) and L62 (*leuc*^l), which differ in phage genotype from the wild type, the phage-free strain CV4 (-B, A, F), which is representative of the two mutants mentioned before, and mutant B1 (*coag*^l) did not differ from the wild type.

We further investigated whether mutants that gave a lower lesion score than the wild type also multiplied less effectively at the site of the injection. In a preliminary experiment with a dose of 1.6×10^6 c.f.u. of strain V4, it was ascertained that multiplication occurred between 4 and 24 h after inoculation. In three further experiments, doses of c. 2.0×10^6 c.f.u. of the wild type and c. 3.0×10^6 c.f.u. of mutant BH5 (α -*lys*^l) were injected into groups of eight mice, and the viable counts in the skin were determined. In the first experiment, the viable counts in the lesions of mutant BH5 (α -*lys*^l) 24 h after injection were lower than those for the wild type (Wilcoxon two-sample test: $p < 0.05$). In the two other experiments, the lower numbers of c.f.u. in the lesions of strain BH5 (α -*lys*^l) after 12 and 24 h were not significantly different from those

TABLE I
Lesion scores 24 h after subcutaneous injection of various doses of the wild type strain V4 and mutants

Strain	Dose	Number of mice challenged	Mean lesion score
V4	2.5×10^6 *	8	2.0
	3.1×10^6 †	24	1.7
	5.1×10^6	8	2.0
BH5 (α - <i>lys</i> ^l)	2.7×10^6 *	8	1.0
	3.9×10^6 †	26	0.9
	2.2×10^7	8	1.0
B21 (α - <i>lys</i> ^l)	2.3×10^6 *	6	1.0
	4.2×10^6 †	6	0.8
	9.0×10^6	6	1.0
BT22 (α - <i>lys</i> ⁻ , <i>leuc</i> ^l)	3.0×10^6 *	8	1.0
BS36 (δ - <i>lys</i> ⁻ , <i>leuc</i> ^l)	2.2×10^6 *	6	0.8
	4.4×10^6 †	6	1.0
	8.2×10^6	4	2.0
CV4 (-B, A, F)	3.0×10^6	6	2.0
B1 (<i>coag</i> ^l)	2.7×10^6	6	2.0

* † The difference in lesion score between the mutant doses marked * and † and the wild type was significant according to the Yates test ($P < 0.001$).

of the wild type ($P < 0.1$ and $P < 0.26$ respectively). In a comparable experiment the viable counts in the lesions of strain BS36 (δ -*lys*⁻, *leuc*^t) did not differ from those of the wild type.

It was concluded from these experiments that production of α -lysin is a necessary condition for the production of dermonecrosis in skin infections caused by *S. aureus*. Because mutant BS36 (δ -*lys*⁻, *leuc*^t), which produced as much α -lysin as the wild type, only gave skin necrosis at higher doses, it seems likely that δ -lysin also contributes to the production of dermonecrosis. The diminished production of leucocidin by this mutant and by mutant BT22 (α -*lys*⁻, *leuc*^t) appeared not to be the reason for the absence of dermonecrosis, because mutant L62 (*leuc*^t) and CV4 (-B, A, F) did not differ from the wild type. In these experiments, it was not possible to decide with certainty whether α -lysin promotes multiplication of bacteria at the site of injection or not.

Intravenous challenge in normal mice

Intravenous injection of *S. aureus* in mice leads to multiplication of bacteria in the kidneys and not in other organs (Gorrill, 1958; Karas and Kapral, 1962). Gorrill (1958) found that, in the dosage range from 1.0×10^6 to 3.0×10^7 c.f.u., there was a linear relationship between the logarithm of the dose and the mortality after 14 days, and between the logarithm of the dose and the number of infected kidneys. When higher doses were injected, some of the mice died within 24 h and the dosage-mortality curve was very steep (Gorrill and McNeil, 1963). In this case, mice probably die as a result of toxin production after a few bacterial divisions.

Mortality and renal abscess formation in intravenously challenged mice. Groups of 15-23 mice, with a mean number of 20, were challenged with doses varying from c. 1×10^7 to 1×10^9 c.f.u. by intravenous injection and the mortality was noted daily. Immediately after death, the kidneys were examined macroscopically for abscess formation. Surviving mice were killed and the kidneys examined at the end of the 14-day experiment. The early mortality (after 2 days) and the total 14-day mortality data given in table II show that the mortality after injection of the wild-type strain V4 increased from 0 to 100% over a short dosage range, so that it was impossible to determine the LD₅₀. The mortality data for 2 and 14 days associated with doses that lay closest to c. 1.0×10^8 and 3.0×10^8 c.f.u. were therefore chosen for comparison (table II). The mutants BH5 (α -*lys*^t), BT22 (α -*lys*⁻, *leuc*^t), BS36 (δ -*lys*⁻, *leuc*^t), BB16 (α -*lys*⁻, δ -*lys*⁻, *dnase*^t, *leuc*^t) and B29 (*coag*^t, α -*lys*⁻, *dnase*^t, *leuc*^t) gave a significantly lower 2-day mortality than the wild type. The 14-day mortality for these strains was also significantly lower ($P < 0.01$), except when the highest dose was given to strain BH5 (α -*lys*^t).

The percentage numbers of renal abscesses produced with doses of c. 1.0×10^8 c.f.u. or higher did not differ significantly and varied from 85 to 100%. Only mutants BT22 (α -*lys*⁻, *leuc*^t), BB16 (α -*lys*⁻, δ -*lys*⁻, *dnase*^t, *leuc*^t) and B29 (*coag*^t, α -*lys*⁻, *dnase*^t, *leuc*^t) appeared to give fewer renal abscesses at the lower doses. For the dose corresponding to 1.2×10^7 of the wild type (lowest dose

TABLE II

Comparison of the percentage numbers of mice dead at 2 and 14 days after intravenous injection of the wild-type strain and mutants

Challenge strain and dose	Percentage numbers of mice dead after	
	2 days	14 days
Wild type V4		
8.8×10^8	100	100
3.1×10^8 *	95	100
1.0×10^8 †	89	94
3.5×10^7	20	35
1.2×10^7	0	0
BH5 (α -lys ^l)		
4.4×10^8 *	40 (P < 0.001)	85
1.1×10^8 †	0 (P < 0.001)	0
1.3×10^7	0	0
BT22 (α -lys ⁻ , leuc ^l)		
8.4×10^8	100	100
3.8×10^8 *	50 (P < 0.001)	70
1.0×10^8 †	15 (P < 0.001)	25
3.4×10^7	0	0
1.3×10^7	0	0
BS36 (δ -lys ⁻ , leuc ^l)		
6.4×10^8	55	95
3.2×10^8 *	10 (P < 0.001)	70
1.6×10^8 †	10 (P < 0.001)	55
4.2×10^7	0	0
1.4×10^7	0	0
BB16 (α -lys ⁻ , δ -lys ⁻ , dnase ^l , leuc ^l)		
7.0×10^8	0 (P < 0.001)	5
2.3×10^8 *	0 (P < 0.001)	0
1.9×10^7 †	0	0
B1 (coag ^l)		
3.3×10^8 *	85 (NS)	95
1.7×10^8 †	79 (NS)	89
3.0×10^7	0	0
9.8×10^6	0	0
B29 (coag ^l , α -lys ⁻ , dnase ^l , leuc ^l)		
1.0×10^9	85	95
3.7×10^8 *	5 (P < 0.001)	10
1.9×10^8 †	0 (P < 0.001)	0
2.0×10^7	0	0
CV4 (-B, A, F)		
9.0×10^7 †	95 (NS)	95
3.0×10^7	5	45
9.9×10^6	0	0

* † The significance (P) of the difference in 2-day mortality between the mutant doses marked * and † and the wild type was determined by the χ -square test. NS = not significant.

mentioned in table II), for example, the percentage numbers were 85 for the wild type, 50 for mutants BT22 and BB16, and 45 for mutant B29.

It may be concluded from these experiments that the lower mortality with mutants BH5 (α -lys^l) and BT22 (α -lys⁻, leuc^l) may be ascribed to non-production or virtual non-production of α -lysin by these strains. The lower mortality with mutant BS36 (δ -lys⁻, leuc^l) appears to be due to the loss of δ -lysin production. Loss of leucocidin production had no influence on the mortality. This follows

TABLE III

Number of colony-forming units (c.f.u.) in the kidneys of 20 mice 24 h after intravenous challenge with wild-type strain and mutants

Experiment number	Strain	Dose	Number of c.f.u. in kidneys	Significance*
1	V4	9.0×10^7	2.8×10^7	P < 0.001 P < 0.01 No difference
	BH5 (α -lys ^l)	1.1×10^8	1.2×10^5	
	BT22 (α -lys ⁻ , leuc ^l)	1.8×10^8	6.5×10^6	
	BS36 (δ -lys ⁻ , leuc ^l)	1.6×10^8	3.7×10^7	
2	V4	1.6×10^8	3.9×10^7	No difference
	BS36 (δ -lys ⁻ , leuc ^l)	1.8×10^8	4.6×10^7	

* Wilcoxon two-sample test.

from the fact that strain CV4 (—B, A, F) gave the same mortality as the wild type despite very little leucocidin production. Mutants BB16 (α -lys⁻, δ -lys⁻, *dnase*^l, leuc^l) and B29 (*coag*^l, α -lys⁻, *dnase*^l, leuc^l), which both have a lower growth rate *in vitro*, gave a lower mortality than the wild type as was to be expected, but it was striking that the percentage of renal abscesses, though somewhat lower at the lower doses, was still appreciable.

Number of viable staphylococci in the kidneys of intravenously challenged mice. The difference in mortality produced by the wild type and the α - and δ -lysin mutants after intravenous injection of c. 1×10^8 – 3×10^8 c.f.u. might be due to the lower production of these toxins during multiplication. On the other hand, one or both toxins may have a growth-enhancing effect *in vivo*. To investigate whether α - and δ -lysin did or did not promote multiplication of the organism in the kidneys, groups of 20 mice were intravenously challenged with a dose of c. 1.0×10^8 c.f.u. Autopsy was performed immediately on the mice that died within 24 h, and the numbers of viable organisms (c.f.u.) in the kidneys (the kidney "viable counts") were determined. The survivors were killed after 24 h and treated in the same way. Moreover, another five mice that had received an injection of the wild type were killed after 30 min. and the kidney viable counts were determined to demonstrate that multiplication did occur in the kidneys. The mean kidney viable count 30 min. after injection of 9.0×10^7 c.f.u. of the wild type was 2.5×10^5 (range 1.1×10^5 – 4.0×10^5). After 24 h the mean count was 100 times greater (see table III). The kidney viable counts for mice challenged with the mutants BH5 (α -lys^l) and BT22 (α -lys⁻, leuc^l) were significantly lower than the counts for mice challenged with the wild type. Results obtained with mutant BS36 (δ -lys⁻, leuc^l) did not differ from those obtained with the wild type in two similar experiments.

It was concluded from these experiments that α -lysin mutants multiply less rapidly in the kidneys of mice, but that this effect is not shown by δ -lysin mutants. α -Lysin thus appears to have a growth-enhancing effect. The difference in mortality between mice challenged with a δ -lysin mutant or the wild type seems to be due only to the lethal activity of δ -lysin.

Intravenous challenge in normal rabbits

It has already been shown that the results obtained on subcutaneous or intravenous injection in mice with the leucocidin mutants L62 (*leuc*^d) and CV4 (— B, A, F) did not differ from those obtained with the wild type. This was not unexpected, because mouse leucocytes, unlike rabbit and human leucocytes, are not sensitive to leucocidin (Gladstone and van Heyningen, 1957). The significance of leucocidin as a virulence factor was therefore investigated by challenging two groups of three rabbits intravenously with the wild type and the mutant L62 (*leuc*^d). After 48 h the rabbits were killed and the numbers of viable organisms (c.f.u.) in the two kidneys were determined separately. One rabbit died after 24 h and was examined immediately. The results given in table IV show that the kidney viable counts after injection of the mutant or the wild type did not differ.

Subcutaneous challenge in irradiated mice

Mice given an injection of 10 c.f.u. or more of *S. aureus* subcutaneously three days after receiving a total body irradiation dose of 750–800 R develop necrotic lesions with massive local bacterial multiplication from which they die (van der Vijver *et al.*, 1974). To determine whether differences in virulence like those found with α - and δ -lysin mutants in normal mice are also detectable in mice with diminished resistance, the wild type and the mutants BH5 (α -*lys*^d) and BS36 (δ -*lys*⁻, *leuc*^d) were injected subcutaneously into groups of 10 mice that had received an irradiation dose of 800 R. The mortality data and the mean lesion scores summarised in table V, show that the mice challenged with the mutants had the same mortality as those challenged with the wild type. The only difference found was that the necrotic lesions caused by mutant BH5 (α -*lys*^d) started 1 day later. This mutant, which never gave necrosis when injected subcutaneously into normal mice, gave necrosis in irradiated mice, probably as a result of the enormous local multiplication (van der Vijver *et al.*, 1974).

It was concluded from these results that differences in virulence such as

TABLE IV
*Number of colony-forming units (c.f.u.) in the kidneys of two groups of three rabbits 48 h after intravenous challenge with wild-type strain and mutant L62 (*leuc*^d)*

Number of c.f.u. 48 h after injection of 2.1 × 10 ⁸ c.f.u. of wild-type strain		Number of c.f.u. 48 h after injection of 1.9 × 10 ⁸ c.f.u. of mutant L62 (<i>leuc</i> ^d)	
in right kidney	in left kidney	in right kidney	in left kidney
6.5 × 10 ⁸	5.6 × 10 ⁸	6.6 × 10 ⁸	6.4 × 10 ⁸
4.6 × 10 ⁸ *	4.4 × 10 ⁸ *	2.8 × 10 ⁸	3.2 × 10 ⁸
4.9 × 10 ⁹	2.9 × 10 ⁸	1.7 × 10 ⁸	4.0 × 10 ⁸

* Rabbit died after 24 h.

have been demonstrated between α - and δ -lysin mutants and the wild type are of no significance when the general resistance of the host has been diminished by irradiation.

DISCUSSION

Mutants with isolated loss of coagulase, staphylokinase or leucocidin production did not differ from the wild type in any of the virulence tests. In other words, it could not be shown that these products are significant for the virulence of *S. aureus* in mice, or that leucocidin is of importance in the virulence of *S. aureus* for rabbits. It therefore appeared to be justified to use the mutant BS36 (δ -lys⁻, leuc^l) to investigate the contribution of δ -lysin to the virulence of *S. aureus* for mice, and the mutant BT22 (α -lys⁻, leuc^l) for such a study of the significance of α -lysin. Leaky mutants with isolated loss of α -lysin were also available for study of the latter factor.

Differences in virulence were found between the wild type and the α - and δ -lysin mutants. The observation that skin lesions in normal mice produced by two α -lysin mutants, which did not differ from the wild type in other respects, never showed necrosis, supports the hypothesis that α -lysin is necessary for the production of necrosis in skin infections. The same conclusion could be drawn from the work of Taubler, Kapral and Mudd (1963) and Noble (1965), but it was not certain that α -lysin alone was responsible for the necrosis. It is therefore noteworthy that a δ -lysin mutant which produced as much α -lysin as the wild type *in vitro*, did not give necrosis until its dosage was increased eight-fold in terms of the minimum necrosis-forming dose of the wild type. Marks and Vaughan (1950) have shown that subcutaneous injection of a crude δ -lysin preparation gave some necrosis of the skin but nothing is known about the activity of δ -lysin *in vivo*. The experiments described here seem to show that dermonecrosis in skin infections in mice caused by *S. aureus* results from production of both α - and δ -lysin.

Crude α -lysin preparations are lethal when injected intravenously in various species of animals. Kapral (1965) has shown that α -lysin probably has a lethal activity during infection; he demonstrated that the LD₅₀ of intraperitoneally injected α -lysin mutants was greater than that of the wild type. In the present

TABLE V

Cumulative mortality (absolute numbers) and mean lesion score (in brackets) in groups of 10 irradiated (800 R) mice subcutaneously challenged with low doses of the wild type and mutants

Challenge strain	Dose	Cumulative mortality and mean lesion score after day*						
		1	2	3	4	6	8	11
V4	34	0 (0)	0 (1.3)	0 (1.6)	2 (1.8)	8 (2.0)	10	10
BH5 (α -lys ^l)	57	0 (0)	0 (0)	1 (1.2)	4 (1.8)	9 (1.8)	10	10
BS36 (δ -lys ⁻ , leuc ^l)	18	0 (0)	0 (0.7)	1 (1.6)	2 (1.6)	8 (1.6)	10	10
... (saline control)	...	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1	10

* Days reckoned from the time of the subcutaneous injection.

study, similar results were found for intravenous challenge. The α -lysin mutants gave a lower early death rate than the wild type when comparable doses were administered. Similar results were also found with a δ -lysin mutant, which produced as much α -lysin as the wild type *in vitro*. The results with the two mutants seem to show that both α - and δ -lysin have lethal activity under the given conditions. The difference in mortality between the α - and δ -lysin mutants and the wild type may be due to reduced production or no production of toxin during multiplication of the bacteria in the kidneys. Alternatively, it may be that the α - or δ -lysin assisted multiplication. An indication that α -lysin has a growth-promoting effect was found in the observation that the number of c.f.u. in skin lesions caused by an α -lysin mutant seemed to be lower than that caused by the wild type. However, the differences were not always significant in repeated experiments. The number of c.f.u. in the kidneys after intravenous injection of two α -lysin mutants, on the other hand, was significantly lower than that caused by the wild type. α -Lysin thus appears to have a growth-promoting effect and can be regarded as an aggressin. A similar growth-promoting effect could not be demonstrated for δ -lysin.

Significant differences in virulence between the wild type and mutants, as demonstrated for α - and δ -lysin mutants in normal mice, could not be found in mice whose general resistance had been lowered by total body irradiation.

It seems reasonable to conclude from the results described here that the significance of the extracellular factors that we have studied for the virulence of *S. aureus* is probably slight, but that α - and δ -lysin can be virulence factors under certain circumstances. Factors with aggressin activity situated in the cell walls (Fisher, 1965; Hill, 1968) are probably more important for maintenance and multiplication of the bacteria in the host. This hypothesis is supported by the observation that differences in virulence were observed, even in irradiated mice, between (i) the "mouse-virulent" strain V4, and (ii) the "mouse non-virulent" strain Wood 46 which lacked the staphylococcal aggressin in the cell wall (van der Vijver *et al.*, 1974).

The suggestion put forward by Blair in 1958, that a combination of factors may be involved in the virulence of *S. aureus*, is supported by this work.

SUMMARY

The contribution of a number of extracellular products of *Staphylococcus aureus* to the virulence of the organism for mice was studied by comparing a wild-type strain with various mutants derived from it in three virulence tests: (1) subcutaneous and (2) intravenous challenge in normal mice, and (3) subcutaneous challenge in mice after total body X-irradiation.

Mutants with lower production or non-production of coagulase, staphylokinase and leucocidin were just as virulent for mice as the wild type in all three tests. Unlike the wild type, mutants with low production or non-production of α -lysin never gave necrosis after subcutaneous injection in normal mice. One mutant with loss of δ -lysin and unaltered α -lysin production gave necrosis only when injected in high doses. Dermonecrosis seems to be caused by a combination of α - and δ -lysin.

Intravenous injection of each of the two types of mutant in normal mice gave a lower mortality rate than that obtained with the wild type. Mutants with deficient α -lysin production, but not δ -lysin-deficient mutants, multiplied more slowly in the kidneys than the wild type under these conditions. α -Lysin appears to have a growth-enhancing effect for the organism *in vivo*, but δ -lysin does not.

Differences in virulence between the wild type and mutants could not be demonstrated in irradiated mice.

REFERENCES

- BLAIR, J. E. 1958. Factors determining the pathogenicity of staphylococci. *A. Rev. Microbiol.*, **12**, 491.
- FISHER, S. 1965. Experimental staphylococcal infection of the subcutaneous tissue of the mouse. III. Further observations on the cellular aggressin of coagulase-producing staphylococci. *J. infect. Dis.*, **115**, 285.
- FISHER, S. AND ROBSON, J. E. 1963. Experimental staphylococcal infection of the subcutaneous tissue of the mouse. I. Bacterial population changes in relation to tissue response. *J. infect. Dis.*, **113**, 204.
- GLADSTONE, G. P. AND VAN HEYNINGEN, W. E. 1957. Staphylococcal leucocidins. *Br. J. exp. Path.*, **38**, 123.
- GORRILL, R. H. 1958. The establishment of staphylococcal abscesses in the mouse kidney. *Br. J. exp. Path.*, **39**, 203.
- GORRILL, R. H. AND MCNEIL, E. M. 1963. Staphylococcal infection in the mouse. I. The effect of route of infection. *Br. J. exp. Path.*, **44**, 404.
- HILL, M. J. 1968. A staphylococcal aggressin. *J. med. Microbiol.*, **1**, 33.
- KAPRAL, F. A. 1965. Factors involved in experimental staphylococcal peritonitis. *Ann. N. Y. Acad. Sci.*, **128**, 259.
- KARAS, E. M. AND KAPRAL, F. A. 1962. Virulence and coagulases of *Staphylococcus aureus*. III. Survival of certain coagulase-negative mutants in the organs of intravenously infected mice. *J. infect. Dis.*, **111**, 209.
- MARKS, J. AND VAUGHAN, A. C. T. 1950. Staphylococcal δ -haemolysin. *J. Path. Bact.*, **62**, 597.
- MILES, A. A., MISRA, S. S. AND IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *J. Hyg., Camb.*, **38**, 732.
- NOBLE, W. C. 1965. The production of subcutaneous staphylococcal skin lesions in mice. *Br. J. exp. Path.*, **46**, 254.
- TAUBLER, J. H., KAPRAL, F. A. AND MUDD, S. 1963. Role of alpha-toxin in lesion formation by *Staphylococcus aureus* on sutures subcutaneously implanted in mice. *J. Bact.*, **86**, 51.
- VIJVER, J. C. M. VAN DER, VAN ES-BOON, M. M. AND MICHEL, M. F. 1974. The enhancing effect of cell-wall aggressin on the virulence of *Staphylococcus aureus* in normal and irradiated mice and in man. *Antonie van Leeuwenhoek*, **40**, 49.
- VIJVER, J. C. M. VAN DER, VAN ES-BOON, M. M. AND MICHEL, M. F. 1975. Induction of mutation in *Staphylococcus aureus* by ethylmethane sulphonate. *J. med. Microbiol.*, **8**, 265.