EXPERIMENTAL STAPHYLOCOCCAL MASTITIS IN THE MOUSE: EFFECTS OF EXTRACELLULAR PRODUCTS AND WHOLE BACTERIAL CELLS FROM A HIGH-VIRULENCE AND A LOW-VIRULENCE STRAIN OF *STAPHYLOCOCCUS AUREUS*

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THE technique of inoculation of staphylococci into the mammary gland of the mouse (Chandler, 1970) has been used to demonstrate that strains of *Staphylococcus aureus* differ in their virulence as assessed by the clinical response, the number of bacteria recovered from the infected glands and the histopathological lesions induced (Anderson, 1971, 1972). The inoculation of cell-free staphylococcal "toxin" i.e., total extracellular products into the mammary gland of the mouse causes death, and the inoculation of diluted "toxin" produces a coagulative necrosis consistent with the action of α -toxin (Anderson and Mason, 1974).

In the present study a comparison was made of the effect of cell-free toxic preparations and live staphylococci derived from two strains. One strain was known to be of high virulence and the other of low virulence in the mammary gland of mice and cattle (Anderson, 1971; Reiter *et al.*, 1971).

MATERIALS AND METHODS

Mice. Lactating mice of the BSVS strain were used between the 3rd and 6th day after parturition; the offspring were removed from their mother 1 hour before the inoculations were given.

Staphylococci. Strains BB and Mexi of staphylococcus were used. Both strains, originally isolated from clinical cases of mastitis in cattle, were coagulase positive and produced α - and β -haemolysins on 5% (v/v) ox-blood agar. Strain BB is more virulent than strain Mexi in cattle (Reiter *et al.*, 1971), and these strains (named C2 and Z2 respectively) exhibited similar relative virulence in mice (Anderson, 1971).

Biochemical tests. Gelatinase activity of each strain was determined in stab cultures in nutrient gelatin (12% w/v gelatin, Oxoid Ltd, Code CM135a) incubated at 37°C for 7 days. Cultures were cooled to 4°C for 30 min. each day and examined for liquefaction.

Each strain was inoculated into litmus milk (Cruikshank, Duguid and Swain, 1965, p. 817), incubated at 37°C, and examined at daily intervals for 7 days for acid coagulation, dye reduction and peptonisation.

Strain BB liquefied gelatin and in litmus milk produced an acid clot with dye reduction but no peptonisation. Strain Mexi failed to liquefy gelatin and produced no reaction in litmus milk.

Production and assay of "toxin". Freeze-dried cultures of the staphylococci were reconstituted by overnight incubation in Hartley's broth (Cruikshank, Duguid and Swain,

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1965, p. 742) and subcultured and incubated overnight in the same medium; 0.1 ml of the broth culture was then seeded on to semi-solid medium (Lominski and Arbuthnott, 1962) dispensed in 20-ml amounts in 100-ml conical flasks and grown for 2 days at 37°C in air with the addition of 20% CO₂. Fluid was harvested from the semi-solid medium by filtration through Green's no. 904½ filter paper, and the filtrate was sterilised by passing it through an asbestos filter (Carlson-Ford Grade HP/EK) under vacuum. "Toxin control" was prepared by adding 0.1 ml of sterile Hartley's broth to the semi-solid medium and processing this as described.

A viable count (Miles, Misra and Irwin, 1938) was made of the organisms of each strain of staphylococcus seeded on to the semi-solid medium for the production of "toxin" and a count was also made of the organisms present in the filtrate after harvesting.

α-Haemolysin was assayed by adding 1 ml of a 1% suspension of washed rabbit RBC to doubling dilutions of toxin preparation and the "toxin control" in saline (0.15M NaCl) to give a final volume of 1 ml. The mixtures were shaken and examined after 1 hour at 37°C. Assay of *β-lysin* was made in the same way except that a 1% suspension of washed sheep RBC was used and the test was read after incubation for 1 hour at 37°C and overnight at 4°C. The end-point was taken as the highest dilution to show 100% haemolysis.

Free coagulase was determined by preparing doubling dilutions of the toxin preparation and the "toxin control" in saline to give a final volume of 0.25 ml. Fresh rabbit plasma (0.25 ml) was added to each tube. The tubes were shaken, incubated at 37°C and observed at hourly intervals for evidence of coagulation. A final reading was made after overnight incubation.

Leucocidal activity was assayed against bovine leucocytes. A suspension of leucocytes was obtained by injecting 0.1 mg of purified *Escherichia coli* endotoxin (Difco) in 20 ml of pyrogen-free distilled water into the mammary gland of a cow via the teat canal and collecting the leucocyte-rich milk after 16–18 hours. The cells were centrifuged at 1000*g* for 20 min., washed three times in sterile saline, and counted in a haemocytometer and adjusted to give a concentration of 5×10^5 cells per ml. Serial doubling dilutions of toxin preparation and "toxin control" were made in saline to give a final volume of 0.25 ml, and 0.25 ml of the leucocyte suspension was added to each tube. After incubation for 1½ hours at 37°C the tubes were shaken and the number of morphologically normal leucocytes in nine large squares of the haemocytometer counted. The leucocidin activity was expressed as the highest titre of toxin preparation containing the same number of cells as the saline control.

Intramammary injection of "toxin". Mice were anaesthetised with ether and 0.1 ml of toxin preparation or control material was inoculated into the fourth mammary gland on each side (R4 and L4) as described by Chandler (1970). Undiluted toxin and toxin diluted 1 in 2 from the two strains were each given to a group of five mice; a fifth group of four mice received "toxin control". The mice were observed for 48 hours, after which the surviving mice were killed. The right mammary gland (R4) from each mouse was fixed in 12% neutral buffered formalin and embedded in paraffin wax. Sections (5 µm) stained with Giemsa's stain and by Gram's method were examined histologically. The left mammary glands (L4) were each ground in a Griffith's tube with 5 ml of sterile saline and checked for aerobic bacterial contaminants.

Intramammary injection of staphylococci. Eighteen-hour subcultures on blood agar of strains BB and Mexi were suspended and washed in sterile saline. The concentration of each strain was finally adjusted to yield approximately 10^{11} organisms per ml. Mice were anaesthetised with ether and 0.1 ml of the appropriate strain of staphylococcus was inoculated into R4 and L4. Twelve mice were used for each strain and four control mice were inoculated with 0.1 ml of sterile saline. The mice were observed for 48 hours, after which time the surviving mice were killed. The R4 mammary glands were fixed and processed for histological examination and the L4 mammary glands ground in a Griffith's tube for viable bacterial-cell counts and, after filtering through Watman's "no. 1 qualitative" filter paper, the filtrate was assayed for *α*- and *β*-lysins. In this experiment the mammary glands of mice that died were also examined as soon as possible after death.

Growth of staphylococci in isolated mammary glands. The inoculum was prepared as

for inoculation into living mice. Ten mice were killed with ether and 0.1 ml of diluted bacterial suspension was inoculated into R4 and L4 immediately after death. The glands were then excised without delay and each was placed in a screw-capped bottle containing 2 ml of sterile saline. Five mammary glands were inoculated with 10^3 and five with 10^{10} staphylococci of each strain. As controls, five mammary glands were inoculated with saline and transferred to sterile saline. After 24 hours the mammary glands were each ground in a Griffith's tube, a viable count was then performed and the filtered homogenate was assayed for α - and β -lysins.

Clinical assessment. A mouse was described as being "malaised" if its coat stood on end and it was not as alert as a normal mouse. The term "ill" was used to describe a mouse with a very poor coat that was disinclined to move.

RESULTS

In-vitro characteristics of the toxin preparations

The viable counts of the staphylococci of strains BB and Mexi that were seeded on to semi-solid medium, the number of organisms in the filtrates and

TABLE I

Characteristics of toxin preparations from Staphylococcus aureus strains BB and Mexi

Strain	Number of staphylococci		Titre† of			
	seeded on to semi-solid agar	per ml of fluid harvested*	α -lysine	β -lysine	free coagulase	leucocidin
BB	1.4×10^9	2.7×10^{13}	1024	32	0	4096
Mexi	1.4×10^{10}	4.1×10^{13}	2048	128	0	4096

* After "coarse" filtration.

† In sterile filtrate.

the in-vitro activity of the toxin preparations from the two strains are shown in table I. The "toxin control" was sterile and had no in-vitro activity.

Effects of intramammary inoculation of toxin preparations in mice

The clinical state of the mice 24 and 48 hours after inoculation of the respective toxin preparations is shown in table II. The effects of the preparations from strain BB and strain Mexi were similar; the "toxin control" produced no ill-effect.

There were extensive areas of coagulative necrosis with karyolysis and peripheral interalveolar infiltration of neutrophils in the mammary glands of mice inoculated with toxin preparations from either strain. No difference in the histopathological response to the two preparations could be detected. Bacteria were not seen in Gram-stained sections and bacteriological examination of ground mammary glands (L4) confirmed their sterility. The mammary glands of mice inoculated with "toxin control" were sterile and showed no histopathological change after 48 hours.

Effects of intramammary inoculation of live staphylococci in mice

The clinical response, number of organisms recovered and toxin assay of each mouse inoculated with strain BB or with strain Mexi are shown in table III, and the difference in virulence between the two strains is apparent. Four mice inoculated with strain BB were dead within 24 hours and a further five died within 48 hours of inoculation. In all of these mice (nos. 1-9) death was associated with the recovery of large numbers of the inoculated organism (geometric mean 4.1×10^{13}) and the presence of α -lysin in the gland. Macroscopically, the inoculated mammary glands of these mice were hard and grey

TABLE II

Clinical state of mice 24 and 48 hours after intramammary inoculation of undiluted or diluted preparations of toxin from Staphylococcus aureus strains BB and Mexi

Strain	Dilution of preparation	Number of mice that after							
		24 hours were				48 hours were			
		dead	ill	"malaised"	normal	dead	ill	"malaised"	normal
BB	Undiluted 1 in 2	5	0	0	0	5	0	0	0
		0	5	0	0	1	0	4	0
Mexi	Undiluted 1 in 2	5	0	0	0	5	0	0	0
		0	5	0	0	1	0	4	0
...*	...	0	0	0	4	0	0	0	4

* "Toxin control"; see text.

in colour, and there was blood-staining of the associated subcutaneous tissue. The clinical response, the macroscopic appearance and the toxin production in the mammary glands of the three surviving mice were correlated with the numbers of organisms recovered.

On the other hand, there were no deaths after the inoculation of strain Mexi; after 24 hours the mice were "malaised", but by 48 hours five of them were clinically normal. The mammary glands of the clinically normal mice were soft and red in colour, while those of the "malaised" mice were firmer in consistency but blood-staining of the subcutaneous tissue was not marked. Neither α - nor β -lysin was found in the mammary glands of the majority of mice inoculated with strain Mexi (nos. 13-20) and this was associated with the recovery of a relatively small number of organisms from them (geometric mean 1.7×10^8). Toxin was detected in the mammary glands of four mice (nos. 21-24) and this was associated with the recovery of a larger number of organisms (geometric mean 5.5×10^{12}). These mice were also clinically and macroscopically more severely affected than nos. 13-20.

Early coagulative changes in the alveolar epithelial cells were seen in the mammary glands of all the mice that were inoculated with strain BB and died within 48 hours. In addition, vacuolation and sloughing of the epithelium

resulted in accumulation of cell debris in the alveolar lumen. There were very few morphologically normal neutrophils in the alveolar lumen and Gram-stained sections showed that most of the staphylococci lay in the lumen free of cells. The mammary gland of mouse no. 11 showed areas similar to that seen in the mice that died as well as areas of classical coagulative necrosis. However, there were many neutrophils in the alveolar lumen of the mammary glands from mice nos. 10 and 12, and there was an epithelial hyperplasia which obscured the alveolar architecture in some areas. In these mammary

TABLE III

Clinical response, numbers of organisms recovered and haemolysins detected in mammary glands 48 hours after inoculation of strains BB or Mexi

Mouse number	After inoculation of strain BB				Mouse number	After inoculation of strain Mexi			
	Clinical state	Number of staphylococci recovered	Titre of			Clinical state	Number of staphylococci recovered	Titre of	
			α -lysin	β -lysin				α -lysin	β -lysin
1	dead*	1.3×10^{13}	4	0	13	normal	3.25×10^8	0	0
2	dead*	1.4×10^{13}	8	0	14	normal	3.8×10^{11}	0	0
3	dead*	1.4×10^{13}	16	0	15	normal	7.8×10^8	0	0
4	dead*	8.3×10^{12}	8	0	16	normal	3.8×10^5	0	0
5	dead	1.4×10^{14}	4	0	17	malaise	2.0×10^6	0	0
6	dead	1.4×10^{14}	8	0	18	normal	3.0×10^6	0	0
7	dead	5.8×10^{13}	4	0	19	malaise	4.3×10^9	0	0
8	dead	1.3×10^{14}	4	0	20	malaise	6.0×10^8	0	0
9	dead	7.3×10^{13}	4	0	21	malaise	9.3×10^{13}	8	2
10	ill	3.7×10^9	4	0	22	malaise	2.3×10^{10}	2	0
11	ill	1.3×10^{14}	8	0	23	malaise	2.4×10^{13}	8	2
12	normal	3.0×10^6	0	0	24	malaise	4.0×10^{13}	8	2

* Twenty-four hours after inoculation.

glands Gram-stained sections showed that there were fewer staphylococci and that most of these were within neutrophils.

There was a marked neutrophil infiltration and hyperplasia of the epithelium that obliterated many alveoli in the mammary glands of the majority of mice inoculated with strain Mexi. Most of the staphylococci were within neutrophils. In the mammary glands of the four mice (nos. 21–24) in which lysins were detected, there were areas of coagulative necrosis with pyknotic but not karyorrhexic nuclei. In these areas most of the staphylococci were extracellular.

No mortality or ill-health, and no histopathological lesions, were seen in the control mice; bacteria were not recovered from the inoculated mammary glands and lysins was not found in the homogenised glands.

Growth of staphylococci in isolated mammary glands

The numbers of staphylococci recovered from isolated mammary glands 24 hours after inoculation (means of five counts) were as follows: with the small inoculum (10^3) of strain BB, 3.8×10^8 , and of strain Mexi, 4.6×10^9 ; and with the large inoculum (10^{10}) of strain BB, 4.2×10^7 , and of strain Mexi,

3.6×10^9 . Culture of the control glands did not yield any staphylococci. Neither α - nor β -lysin was detected in the filtrate of any of the homogenised mammary glands.

DISCUSSION

Preparations of toxin from the two staphylococcal strains were alike in their in-vitro properties and in their effect on the mammary glands of mice, but when live cells of each strain were inoculated into the gland strain BB was more virulent than strain Mexi. This suggests that the cells of the strain Mexi were capable of elaborating toxins similar in quality and potency to those from strain BB, but that either strain Mexi was prevented from expressing its virulence *in vivo* or strain BB possessed some factor that allowed it to express its virulence *in vivo*.

The illness and mortality resulting from the intramammary inoculation of cell-free staphylococcal toxins established a connexion between "toxin" and clinical response. By inoculation of various dilutions of "toxin" a "titration effect" can be seen both clinically and histopathologically (Anderson and Mason, 1974). At least some of the more severe responses seen after the inoculation of live staphylococci may therefore be attributed to the production of toxic substances *in vivo*. Experiments *in vivo* and *in vitro* have established that staphylococcal "toxin" is liberated during the late logarithmic stage of bacterial growth (Gladstone and Glencross, 1960; Duncan and Cho, 1971). The clinical and histopathological lesions seen in response to inoculation of strain BB into mice were associated with the multiplication of that strain in the mammary gland and the presence in it of α -lysin; the absence of severe clinical and histopathological lesions in mice inoculated with strain Mexi was associated with the relative failure of that strain to multiply in the mammary gland of the majority of mice, and this in its turn was associated with the absence of detectable α -lysin in the gland (table III).

The failure of a strain of staphylococcus to multiply in the mammary gland might be due to an inadequacy of the gland as a medium for bacterial multiplication, or to the susceptibility of the strain to the bacterial defence mechanism of the host. However, my observations demonstrated that mammary gland tissue was not a nutritionally inadequate medium for the growth of either strain. It therefore seemed that strain Mexi was more susceptible to the host defence mechanism or, conversely, that strain BB possessed some factor which made it resistant to bactericidal mechanisms and thus able to multiply unimpaired.

Chesbro, Wamola and Bartley (1969), who studied staphylococcal strains from bovine mastitis, concluded that the rapidity of their growth in batch and continuous culture could be correlated with their virulence. They implied that the inherent ability to grow more rapidly and thus outpace the host defence mechanism distinguished the high-virulence from the low-virulence strains; but *in vivo* the resistance of a strain to phagocytosis and killing may also contribute to a rapid increase in bacterial numbers. If so, the intramammary inoculation of a large dose of strain BB would be followed by only a slight

fall in number and then by rapid growth, whereas a similar inoculum of strain Mexi would be greatly reduced in number before multiplication began. The results presented give no information about the kinetics of bacterial multiplication during the infection, but a difference in susceptibility to bactericidal mechanisms would be expected to affect the rate of release of toxin. Kapral Keogh and Taubler (1965) showed that, when staphylococci were grown in dialysis sacs in the peritoneal cavity of mice, the growth of a large inoculum resulted in the rapid formation of large amounts of toxin but that multiplication of a smaller inoculum resulted in a gradual formation of toxin. In my experiments, strain BB multiplied rapidly with elaboration of toxin so that four of 12 mice were dead within 24 hours, large numbers of bacteria were recovered, and toxin was detected in the mammary gland. Histopathological examination showed that there was extracellular multiplication of bacteria, destruction of neutrophils and early coagulative necrosis. Within 48 hours a further five mice were dead with similar necropsy findings. In contrast, strain Mexi appeared to multiply slowly in the mammary gland either without detectable toxin production or with only a gradual release of toxin. Epithelial hyperplasia and intracellular staphylococci, which were not features of the response to strain BB, were the predominant histopathological findings in the mammary glands of mice inoculated with this strain.

In the exceptions among the mice that received strain Mexi (mice nos. 21-24), the host defence-mechanism was presumably less effective against that strain, allowing a more rapid multiplication of organisms with resultant toxin formation and coagulative necrosis. In this respect these mice resembled mouse no. 11 that received strain BB and survived. The histopathological changes in the mammary glands of the two other surviving mice that received strain BB were similar to those in the majority of mice that received strain Mexi; this was probably due to a greater resistance to the challenging strain. Thus the biological variation in the resistance of mice may have created an "overlap effect" in the response to the two strains.

The fact that strain BB liquefied gelatin and reduced litmus milk while strain Mexi did not is in complete agreement with the finding of Chesbro *et al.* (1969) who reported that 72% of their high-virulence strains both liquefied gelatin and reduced litmus milk, while only 16% of low-virulence strains gave positive results in both reactions.

SUMMARY

Cell-free preparations of "toxin" were made from two strains of *Staphylococcus aureus*, one known to be of high virulence and the other of low virulence in the mammary gland of cattle. These preparations were similar in their in-vitro properties and in their clinical and histopathological effect when inoculated into the mammary gland of mice. However, when live staphylococci were inoculated into the mammary gland of mice a difference in virulence corresponding to that seen in cattle was detected by the clinical response, and by the histopathological appearance, the number of organisms, and the amount of α -lysin present in the infected glands. The reasons for

the failure of the low-virulence strain to express its potential virulence are discussed.

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