J. Hyg., Camb. (1979), **83**, 103
Printed in Great Britain

The effect of an intramammary infusion of endotoxin on the establishment of experimental mastitis by *Streptococcus agalactiae* in the cow

By J. BROWNLIE

Agricultural Research Council, Institute for Research on Animal Diseases, Compton, Newbury, Berks.

(*Received* 6 *September* 1978)

SUMMARY

An infusion of *E. coli* endotoxin (10 μg) into the mammary gland of the cow 16 h before experimental infection with *St. agalactiae* prevented the establishment of mastitis. A clinical examination of the affected gland did not reveal signs of inflammation, all organisms were eliminated from the milk by 36 h after infection. Mammary glands not pretreated with endotoxin, and injected with the same number of viable streptococci, showed signs of severe inflammation, the milk yield was reduced and the injected pathogen could be isolated from the gland for up to 14 days when sampling was discontinued. The injection of endotoxin alone produced a rapid increase in the cell count of the milk, there were some signs of inflammation and 80% of the cells in the milk were neutrophils. The cell counts in the milk remained above normal for 7–10 days. Untreated control quarters appeared to be unaffected by the injection of *E. coli* endotoxin into an adjacent quarter, on the other hand the injection of *St. agalactiae* into the adjacent quarter on the same side of the udder produced a cell response which reached a peak after 2 days and returned to preinoculation levels by the fourth day. No inflammation was observed in these control quarters and no streptococci were isolated from the milk.

INTRODUCTION

It has been known for some time that endotoxins can increase the resistance of laboratory animals to infection (Rowley, 1955). In cattle they are reported to cure certain chronic *Pseudomonas aeruginosa* mastitis infections, when given in large amounts, up to 16 mg, directly into the mammary gland (Schalm & Ziv-Silberman, 1968). However, there are serious disadvantages with high doses of endotoxins, they cause considerable local inflammation which can be observed clinically and, in some cases, a systemic reaction that can result in death.

These experiments investigate the effect of microgram amounts of endotoxin on the establishment of experimental infection with *Streptococcus agalactiae* in the mammary gland.

0022–1724/79/8 0108–1978 $01.00

MATERIALS AND METHODS

Animals

The six cattle used were 2- to 4-year old Friesians in the first 8–12 weeks of their first or second lactation. They were housed in isolation units and fed on a conventional diet of hay, concentrates and water. They had no history of mastitis and their milk cell counts were below 100 000 cells/ml of milk, of which the neurophil count was less than 10%. No pathogenic bacteria were isolated from milk samples that had been taken with full hygienic precautions.

Bacteria

A strain of *Streptococcus agalactiae* (Code No. S13) was obtained from the Compton collection of mastitis pathogens. For the experimental production of mastitis, a culture of the S13 strain was grown for 18 h in 10 ml of nutrient broth. This gave a total viable count of 2×10^8 organisms and was the challenge dose for individual quarters. The washed organisms, suspended in 10 ml of saline, were infused into the mammary gland by a sterile cannula through the teat canal.

Endotoxin

Ten μg of bacterial endotoxin (Bactolipopolysaccharide B from *Escherichia coli* O55:B5 (Difco Laboratories, Detroit, U.S.A.) was dissolved in 20 ml of pyrogen-free distilled water and infused by a sterile cannula through the teat canal of individual quarters.

Cell counting

Total and differential cell counts were made on milk smears fixed and stained by the 'single dip' technique of Broadhurst & Paley (1939).

Production of experimental mastitis

The experimental design was the same in all cattle. Two quarters, the right fore and right hind, were infused with endotoxin after completion of the afternoon milking. After the next morning's milking, 16 h after endotoxin treatment, the challenge dose of *St. agalactiae* S13 was infused into the left and right hind quarters. The remaining quarter, the left fore quarter, acted as a control, having had neither endotoxin nor *St. agalactiae* inoculation.

Fore milk samples were taken, with full sterile precautions, from all quarters at the morning and afternoon milking. Sampling started 2 days before endotoxin treatment and continued for at least 12 days.

The bacteria in the milk were counted by the Miles, Misra & Irwin (1938) method. The streptococcal counts were made on Edwards' medium and the total bacterial counts on washed blood agar plates. The results are expressed as the number of organisms/ml of milk. In all experiments the contaminants count was insignificant with numbers below 10^2/ml.

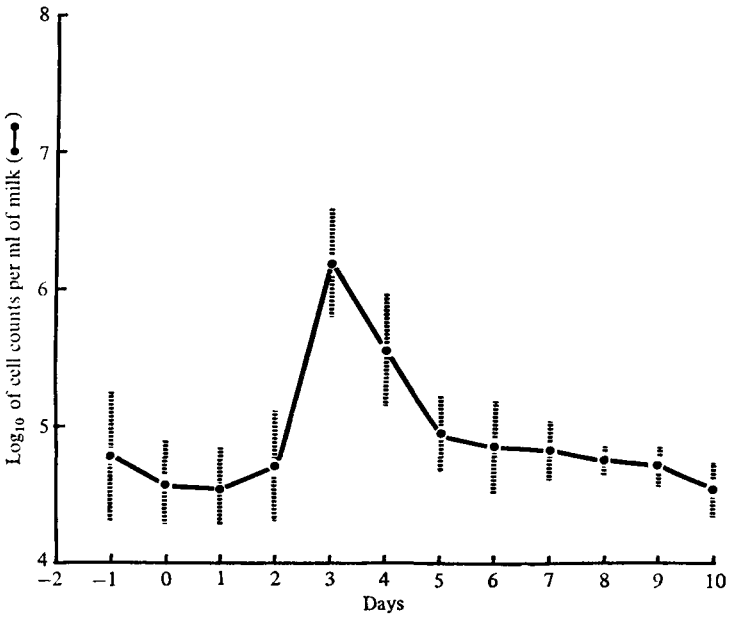

Fig. 1. The left forequarters had no treatment. Each point is the mean (± S.E.M.) value of milk cell counts obtained from the left forequarters of six cows.

RESULTS

Control quarters

The infusion of low doses of endotoxin into the two right quarters on day 0 appeared to have no effect on the control glands during the 16 h between inoculation and challenge with *Streptococcus agalactiae*. This lack of effect has been seen in other unpublished experiments where the cell counts were continued for 72 h. The cell response observed on day 2 in the control quarters followed the infection of the two hind quarters with streptococci. This cell response reached a mean value of 1×10^7 on day 3 and returned to the preinoculation level by day 5 (Fig. 1). No inflammation was observed in these control quarters and no streptococci were isolated from the milk.

Endotoxin-treated quarters

There was a rapid cell response in the milk from all quarters treated with endotoxin (Fig. 2). By 16 h the mean cell count had reached $2–5 \times 10^7$ and continued above 10^7 cells/ml for 3 days, subsiding to normal levels by 7–10 days. Clinical examination of the quarters demonstrates little gross inflammation but differential counts of the milk showed that the neutrophil response was above 80% for at least 5 days after endotoxin infusion. There were no clots in the milk and *St. agalactiae* was not isolated.

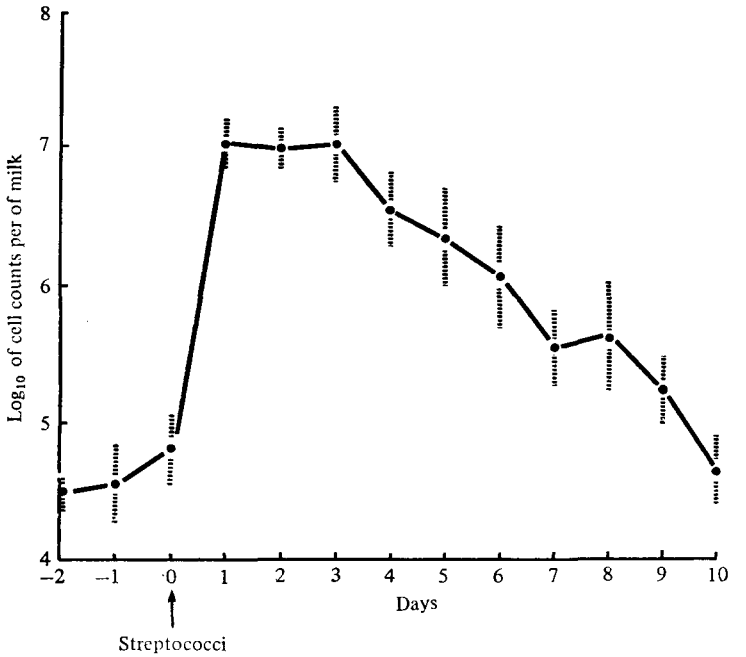

Fig. 2. The right forequarters were inoculated with 10 μg of endotoxin on day 0. Each point is the mean (± S.E.M.) value of milk cell counts obtained from the right fore quarters of six cows.

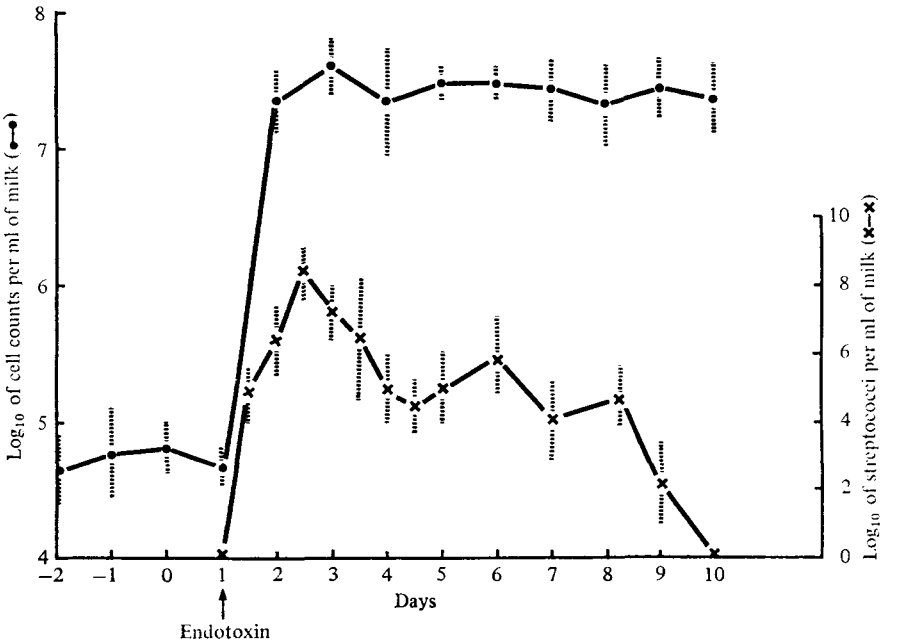

Fig. 3. The left hind quarters were inoculated with 2×10^8 viable *Streptococcus agalactiae* organisms on day 1. Each point is the mean (± S.E.M.) value of milk cell counts (●) and viable streptococci (×) obtained from the left hind quarters of six cows.

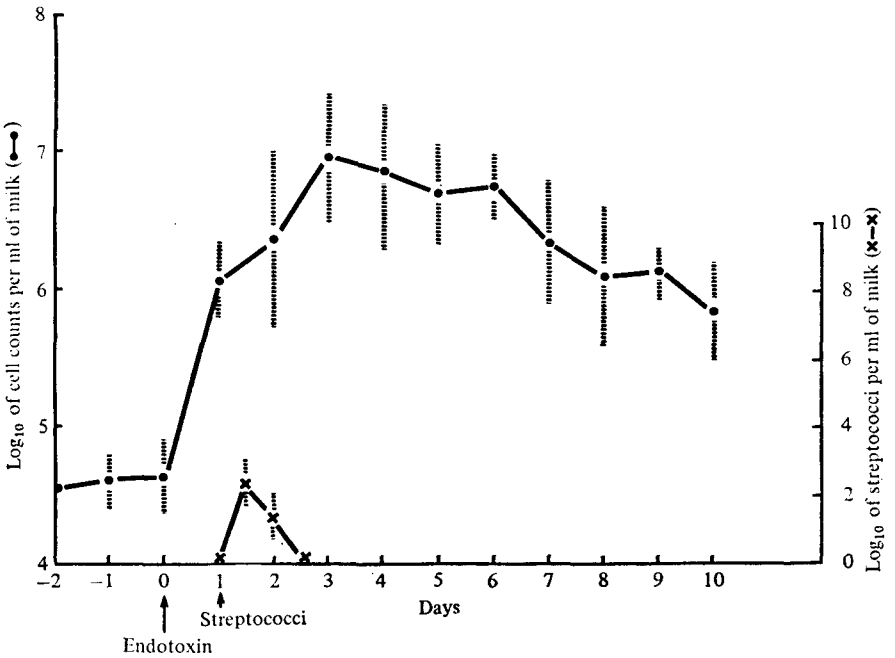

Fig. 4. The right hind quarters were inoculated with 10 μg of endotoxin on day 0, and 2×10^8 viable *Streptococcus agalactiae* organisms on day 1. Each point is the mean ($\pm$ S.E.M.) value of milk cell counts (●) and viable streptococci ($\times$) obtained from the right hind quarters of six cows.

St. agalactiae-*inoculated quarters*

Inoculation of large numbers of viable streptococci, 2×10^8 organisms, into the left hind quarters produced a noticeable clinical response for about 48 h. The inoculated quarters were swollen and inflamed. The milk was clotted in all of the cows and the mean cell counts approached 10^8 cells/ml (Fig. 3). The milk yield from the infected quarter was reduced and in some quarters, the numbers of *St. agalactiae* was as high as 2×10^9/ml although the mean was 6×10^8/ml (Fig. 3). The mean cell response remained above 10^7/ml milk for the 10 days of the experiment, of which over 80% of the cells were neutrophils. Streptococci could not be recovered on day 10 or onwards as far as day 14 when sampling ceased.

Endotoxin and St. agalactiae-*inoculated quarters*

Fig. 3 showed that endotoxins alone can produce a pronounced cell response by 16 h. If, at 16 h, streptococci were inoculated, the cell response continued to resemble that produced by endotoxin alone and not that following a *St. agalactiae* inoculation (Fig. 4). The difference between quarters treated with only endotoxin, and endotoxin-treated quarters challenged with *St. agalactiae*, appeared after day 4 when the return of cell counts to preinoculation levels was slower (10 days), in the latter quarters.

A clinical examination of the affected gland did not reveal noticeable heat or swelling and in only two milk samples from one of the six cows were clots recovered in the milk. There was a recovery of low numbers of *St. agalactiae* in the two milkings following inoculation, but only one cow had streptococci in its milk at the third milking at 76 h. Beyond 36 h no streptococci were detected in the milk.

DISCUSSION

This report demonstrates that microgram amounts of endotoxin can be effectively used *in vivo* to prevent the establishment of experimental infection with 2×10^8 *St. agalactiae* organisms. It is the first report in cattle that such small doses of pure endotoxins have been used for this purpose, although the principle of stimulating a pre-existing leukocytosis to prevent infection has been reported (Schalm, Lasmanis & Carroll, 1966, 1967). The protective effect of endotoxins can be partly explained in terms of the leukocytosis in milk but they are able to promote various other responses (Neter, 1969). They are known to increase the amount of antimicrobial cationic proteins in milk that are active against streptococci (Brownlie, 1971). They are mitogenic for the antibody-forming cells (Peavy, Adler & Smith, 1970) and can catalyse the complement system (Mergenhagen *et al.* 1969). The general stimulation of these mechanisms and certain other non-specific mechanisms (Hibbitt & Hill, 1977) may play an added role, above that of the leukocytosis, in the protection of the mammary gland. This role of stimulating non-specific mechanisms may be even more important if the report, that neutrophils from cow's milk have a reduced ability to kill *Staphylococcus aureus* (Russell, Brooker & Reiter, 1976), were to be extended to other bacteria.

The protective effect of endotoxins in this work appears to be confined to the inoculated quarter. There was no evidence of protection or cell responses in the contralateral quarters. Therefore, the cell response observed in control quarters following *St. agalactiae* inoculation in collateral quarters, is all the more interesting, particularly as streptococci were not detected in the control quarters. The reason for this could be that left and right sides of the mammary gland are divided by a double septum, whereas there is no well-defined separation between fore and hind quarters on the same side (Sisson & Grossman, 1938). In the design of these experiments it will be noted that endotoxin was injected into the quarters on the right side, but streptococci were injected into the hind quarters on both sides. Thus, there may be a possibility of inflammatory products diffusing between quarters on each side following streptococcal inoculation either directly or via shared lymphatics. It has been demonstrated that 77% of lymphatics draining one side of the mammary gland enter the same draining lymph node on that side (El Hagri, 1945). However, in the non-inflamed gland, dyes have not been shown to diffuse between quarters on the same side (Sisson & Grossman, 1938).

The possibility of using endotoxins in a preventive role to stimulate non-specific resistance must be accepted with extreme caution as endotoxins themselves cause local inflammation even when used in microgram amounts. Further studies

with lower doses of endotoxin would be necessary to see what correlations exist between inflammation and protection.

The author wishes to thank Mrs M. Gleed for skilled technical assistance.

REFERENCES

BROADHURST, J. & PALEY, C. (1939). A single-dip stain for the direct examination of milk. *Journal of the American Veterinary Medical Association* **94**, 525–6.

BROWNLIE, J. (1971). Antimicrobial proteins in bovine neutrophils. *Biochemical Journal* **125**, 81–2.

EL HAGRI, M. A. A. M. (1945). Study of the arterial and lymphatic systems in the udder of the cow. *Veterinary Journal* **101**, 27–33, 51–63.

HIBBITT, K. G. & HILL, A. W. (1977). Non-specific resistance to infection in relation to mastitis. In *Antibodies and Antibiosis in Agriculture* (ed. M. Woodbine), p. 245. London: Butterworths.

MERGENHAGEN, S. B., SYNDERMAN, R., GEWURZ, H. & SHIN, H. S. (1969). Significance of complement to the mechanism of action of endotoxin. *Current Topics in Microbiology and Immunology* **47**, 37–77.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene* **38**, 732–49.

NETER, E. (1969). Endotoxin and the immune response. *Current Topics in Microbiology and Immunology* **47**, 82–124.

PEAVY, D. L., ADLER, W. H. & SMITH, R. T. (1970). The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. *Immunology* **105**, 1453–8.

RUSSELL, M. W., BROOKER, B. E. & REITER, B. (1976). Inhibition of the bactericidal activity of bovine polymorphonuclear leucocytes and related systems by casein. *Research in Veterinary Science* **20**, 30–35.

ROWLEY, D. (1955). Stimulation of natural immunity to *Escherichia coli* infections. Observations in mice. *Lancet* i, 232.

SCHALM, O. W., LASMANIS, J. & CARROLL, E. J. (1966). Significance of leucocyte infiltration into the milk in experimental *Streptococcus agalactiae* mastitis in cattle. *American Journal of Veterinary Research* **27**, 1537–46.

SCHALM, O. W., LASMANIS, J. & CARROLL, E. J. (1967). Experimental *Streptococcus agalactiae* mastitis in cattle: Attempts to superimpose the organism in lactating glands harboring unrelated bacterial infections and in glands with experimentally induced sterile inflammation. *American Journal of Veterinary Research* **28**, 685–695.

SCHALM, O. W. & ZIV-SILBERMAN, G. (1968). Reactions following intramammary infusions of *E. coli* endotoxin. *Veterinary Record* **82**, 100–103.

SISSON, S. & GROSSMAN, J. D. (1938). *The Anatomy of the Domestic Animals*. Philadelphia: W. B. Saunders.