INFECTION AND IMMUNITY, Aug. 1977, p. 250-256
Vol. 17, No. 2

Printed in U.S.A.

Effect of Immunization with Highly Purified Alpha- and Beta-Toxins on Staphylococcal Mastitis in Rabbits

C. ADLAM,* P. D. WARD, A. CHRISTINE McCARTNEY,[1] J. P. ARBUTHNOTT,[2] AND C. M. THORLEY

Department of Bacteriology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, England, and Department of Bacteriology, Royal Infirmary, Glasgow G40 SF, Scotland*

Received for publication 25 January 1977

Experiments were carried out to determine whether immunization of female rabbits with highly purified staphylococcal alpha- or beta-toxins would protect them against intramammary challenge with staphylococci. High circulating anti-alpha-toxin titers reduced the lethal hemorrhagic edematous form of the disease ("blue-breast") produced by strains BB and Compton 201 to a localized chronic abscess form. No such protection was afforded by high anti-beta-toxin titers. Immunization with alpha- or beta-toxins produced no change in the clinical picture of the disease produced by CN.6708, a strain of *Staphylococcus* responsible for a natural outbreak of abscess-type rabbit mastitis. From these experiments it would appear that alpha-toxin is a key antigen in the blue-breast form of rabbit mastitis. Since the abscess form of the disease was not prevented by immunization with either alpha- or beta-toxin, other virulence factors must be acting to produce this more localized disease.

The lactating mammary gland of the rabbit is susceptible to natural infection by staphylococci. Two types of disease occur. The first, which is rarely fatal, is typified by the development of pus-filled abscesses under or near one or more teats (3, 20, 26). The second, sometimes called "blue-breast," is a spreading condition in which the mammary tissue becomes edematous and hemorrhagic (8, 17, 22). It is rapidly fatal.

In a previous study we showed that both types of mastitis could be reproduced in the laboratory either by injecting low numbers of organisms into the mammary tissue or by colonizing suckling young with broth-grown organisms (3). The type of disease produced varied with the strain used to infect. Using crude, inactivated, whole culture vaccines, protection against blue-breast was obtained. Immunized does challenged with strains that normally produced blue-breast developed instead the localized abscess disease (2).

In the present work, highly purified alpha- and beta-toxins have been used as immunizing antigens in an attempt to reproduce the protection previously obtained and hence to identify the toxin(s) responsible for the blue-breast condition.

[1] Present address: Department of Bacteriology and Immunology, Western Infirmary, Glasgow G11 6NT, Scotland.
[2] Present address: Department of Microbiology, Moyne Institute, Trinity College, Dublin 2, Ireland.

MATERIALS AND METHODS

Staphylococcal strains. Staphylococcal strains were maintained freeze-dried in the Wellcome Culture Collection. Their culture numbers and sources were: Wellcome CN.6708 (naturally occurring rabbit mastitic abscess); Compton 201 (bovine mastitis); BB (bovine mastitis); Wood 46 (National Collection of Type Cultures no. NCTC 7121).

Organisms were cultured in broth and prepared for infection experiments as previously described (3). Phage typing was kindly carried out for us by J. Edmonds at the Bacteriology Department, Queen Elizabeth Hospital, Birmingham, England.

Alpha-toxin. A crude culture supernatant from Wood 46 was prepared by the method of Bernheimer and Schwartz (7), precipitated with 85% saturated ammonium sulfate, and purified by isoelectric focusing. A pH gradient of 3 to 10 stabilized in a sucrose density gradient, using the LKB 8102 column, was employed (18). The main alpha-toxin component obtained was refocused using the LKB 8101 column, and the peak fraction (isoelectric point, 8.55) was retained and stored as an ammonium sulfate slurry.

When required, this slurry was centrifuged (20 min, 39,000 × *g*), and the pellet was dissolved in 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8) before dialyzing against two changes of this buffer. Alpha-toxin could be stored in this form at −40°C for a limited time.

Beta-toxin. Strain BB was grown in a 4-liter culture vessel with continuous stirring (500 rpm) for 7 h at 37°C using a growth medium of bullock heart infusion (Wellcome) containing 20% (vol/vol) caseinate digest and 0.5% glucose. After centrifugation (1 h, 2,500 × *g*), crude beta-toxin was precipitated from the supernatant with 85% saturated ammonium sul-

fate and subjected to isoelectric focusing in a pH gradient of 3 to 11 stabilized in a sucrose density gradient using an LKB 8102 column. The main beta-toxin component was refocused under the same conditions using an LKB 8101 column, and the peak fraction of beta-toxin (isoelectric point, 9.30) was collected and dialyzed against 0.03 M borate buffer at pH 8.3. It was found necessary to freeze purified beta-toxin at −40°C for storage since ammonium sulfate at concentrations up to 90% saturation was not effective in precipitating the toxin.

Purity and hemolytic activity of the toxins. The purity of both toxins was assessed by sodium dodecyl sulfate-polyacrylamide discontinuous gel electrophoresis (18). Immunodiffusion and immunoelectrophoresis (24) were carried out by running the preparations against their respective antisera and against commercial horse antisera (Wellcome). The preparations were also checked for absence of the following biological activities: tributyrinase (25), egg yolk factor (9), fibrinolysin (27), free coagulase (10), phosphatase (10), and gelatinase (15). The absence of contaminating hemolysins was confirmed by using the appropriate erythrocytes.

Hemolytic activity was assessed by titration. Doubling dilutions of the purified materials were mixed with equal volumes of thrice-washed erythrocytes (rabbit for alpha-toxin, sheep for beta-toxin, and horse and human for delta-toxin) in glass tubes. End points were assessed after incubation (37°C for 30 min followed by overnight at 4°C). Titers were recorded as the reciprocal of the dilution of toxin in the last tube showing lysis. The diluent used for alpha- and delta-toxin estimations was phosphate-buffered saline (pH 7.2) and for beta-toxin, Tris-buffered saline (pH 7.4) containing 10 mM magnesium chloride and 0.1% (wt/vol) bovine serum albumin (Armour, Pharmaceutical Co. Ltd., Eastbourne, Sussex, England).

Vaccine formulation and application. For immunization, the toxin preparations were diluted in Tris-hydrochloride buffer and, in the case of the alpha preparation, toxoided with dilute glutaraldehyde (21) until no erythrocyte lysis was detectable (approximately 90 min). Alhydrogel (Superfos Export Co., Copenhagen, Denmark) was then added (1 part to 4 parts of antigen). The final concentration of the antigens in alhydrogel was 10,000 minimal hemolytic doses/ml (150 μg of protein per ml as estimated by 280-nm absorption for the alpha-toxin; 6 μg of protein per ml for the beta-toxin).

Rabbits were immunized as follows. Antigen on alhydrogel was emulsified with an equal volume of complete Freund adjuvant (Wellcome), and 0.75 ml was injected intramuscularly into both rear legs. These injections were repeated after 1 week. Six weeks after the second dose, animals were injected intravenously twice weekly for 3 weeks with 0.2-ml volumes of antigen on alhydrogel without complete Freund adjuvant. The immunization schedule and artificial insemination of rabbits (see below) were timed so that the final injection was given 3 to 5 days before parturition (10 to 14 days before infection).

Assays for antibodies in serum and milk. Blood from the marginal ear vein was allowed to clot, and serum was removed and heat inactivated (56°C for 1 h) prior to assay. Milk was obtained from does in lactation after intravenous administration of oxytocin (Pitocin, Parke Davis & Co., Pontypool, Mon. Wales; 0.3 U/rabbit). The mammary tissue was massaged with the fingers, and ejected milk was collected into screw-capped bottles. On centrifugation (1 h, 105,000 × g), the milk separated into three layers. Both the upper cream layer and the lower layer of cells were discarded. The middle layer was retained for assay. The method for estimating agglutinating antibodies and antibodies to alpha- and beta-toxins and coagulase have been previously described (3). Antitoxin titers are expressed in international units per milliliter.

Infection experiments. Groups of four to six Californian strain does (Hyline Rabbits Ltd., Marston, Northwick, Cheshire, England) were artificially inseminated by the method of Adams (1). Eight to 10 days after parturition, they were infected by injection of viable organisms at the base of four teats. Two teats on each rabbit received a high challenge and two received a low challenge. The numbers of organisms used were: strain CN.6708, 1.2×10^4 and 3×10^3; strain BB, 8.8×10^3 and 2.2×10^3; and strain Compton 201, 2×10^3 (experiment 1) or 1.4×10^3 (experiment 2). Infection was monitored daily for 8 days by measuring the areas of blue discoloration and thickening in the mammary tissue. The identity of an infecting organism was confirmed by plating out infected tissues. Isolated organisms were checked for pigment, hemolytic pattern, lipase, free coagulase, bound coagulase, and phage-typing pattern for comparison with the infecting inoculum.

RESULTS

Purity of the alpha-toxin. In this kind of investigation, it was important to ensure that the immunizing antigens used were pure. By sodium dodecyl sulfate-polyacrylamide discontinuous gel electrophoresis, the alpha-toxin appeared as two bands (Fig. 1). Previous work has shown the major band to be the 3S component of molecular weight 36,000 and the minor band to be the 12S aggregate of molecular weight 170,000 (5).

In immunodiffusion experiments, the alpha-toxin produced a single precipitin line when tested against autologous rabbit antisera (Fig. 2) or with crude horse antiserum (Wellcome RX.5275) raised against inactivated crude staphylococcal culture filtrates (Fig. 3). Crude culture filtrate containing alpha-toxin also gave a single line when tested against the rabbit antisera (Fig. 4). When similar immunoelectrophoresis experiments were carried out, the formation of these single lines was confirmed.

No tributyrinase, egg yolk factor, fibrinolysin, free coagulase, phosphatase, gelatinase, beta-toxin, or delta-toxin was detected by the

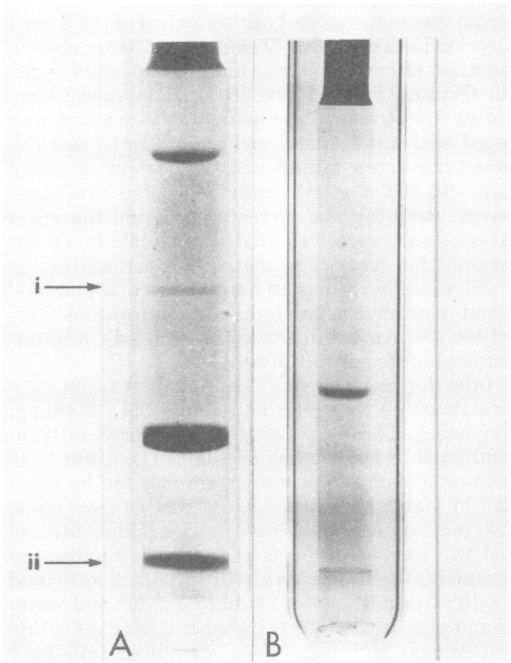

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide discontinuous gel electrophoresis profile of alpha-toxin and beta-toxin. (A) Alpha-toxin; (B) beta-toxin. (i) Artefact band; (ii) track dye band.*

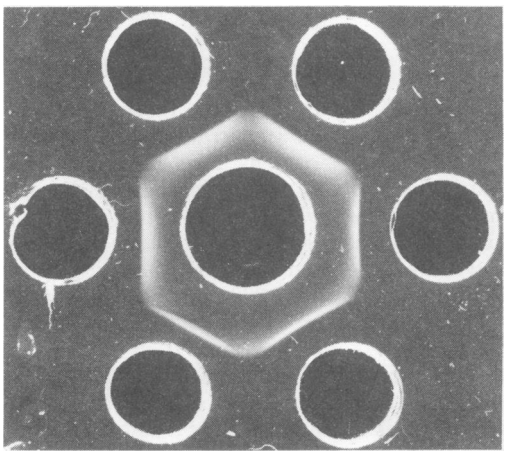

FIG. 2. *Immunodiffusion slide. (Center well) Purified alpha-toxin. (Peripheral wells) Sera from rabbits immunized with purified alpha-toxin.*

biological assays described under Materials and Methods.

Purity of the beta-toxin. When the beta-toxin preparation was tested by sodium dodecyl sulfate-polyacrylamide discontinuous gel electrophoresis (Fig. 1), there was one band of molecular weight 29,000 to 30,000.

In immunodiffusion experiments, the beta-toxin gave a single precipitin line when tested against sera from rabbits immunized with it or against crude horse antiserum (Wellcome EX.1433) containing antibody to beta-toxin (Fig. 5). Identical results were obtained using immunoelectrophoresis.

Elicitation of antibody. Both toxin preparations generated a satisfactory antibody response, although individual rabbits varied more in their response to the beta-toxin than to the alpha-toxin (Fig. 6 and 7). In an experiment in which rabbits immunized with beta-toxin were milked and bled prior to challenge, the antibody level in the milk was between 5 and

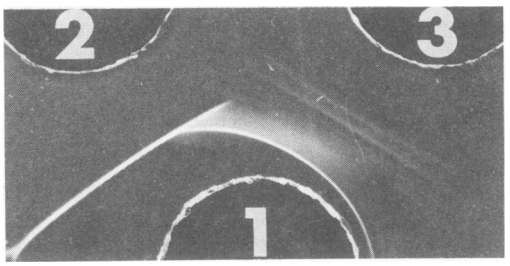

FIG. 3. *Immunodiffusion slide. (Well 1) Crude horse antiserum RX.5275; (well 2) purified alpha-toxin; (well 3) crude Compton 201 culture supernatant.*

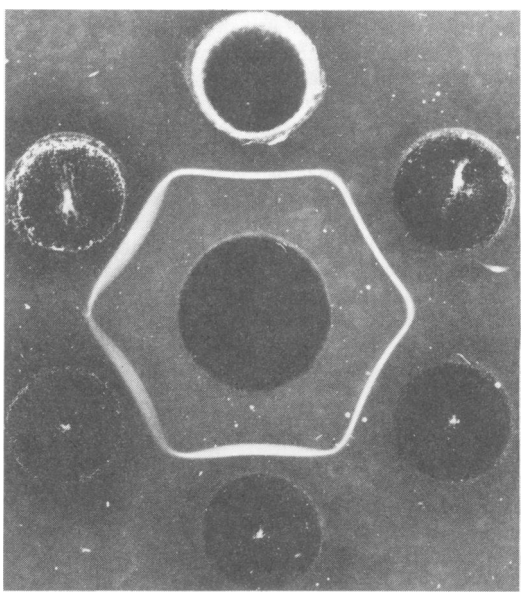

FIG. 4. *Immunodiffusion slide. (Center well) Crude Compton 201 culture supernatant containing alpha-toxin. (Peripheral wells) Sera from rabbits immunized with purified alpha-toxin.*

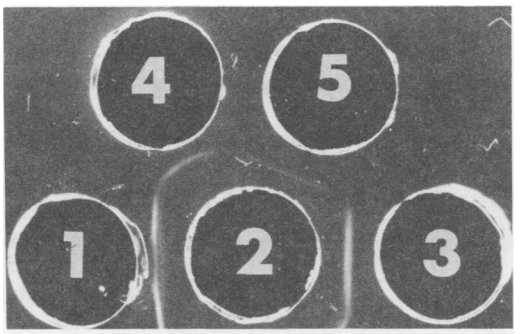

FIG. 5. *Immunodiffusion slide. (Well 2) Purified beta-toxin; (wells 1, 3, 4) sera from rabbits immunized with purified beta-toxin; (well 5) crude horse antiserum EX.1433.*

10% of that in the sera (Table 1). Antisera raised against either the alpha or beta preparation did not neutralize coagulase or cause agglutination of staphylococci of strain BB and CN.6708 to any significant extent. Agglutination of Compton 201 cells was, however, produced by both preimmune sera and sera from immunized animals. The reason for this remains unknown. Titers between animals varied from 1/160 to 1/5,120. No increase in agglutinating titer of the sera was noted after vaccination with either the alpha or beta preparation. No cross-neutralization between the hemolysins and their antisera was seen.

Challenge experiments. The results of two challenge experiments are shown in Fig. 6 and 7. Compton 201 and BB strains produced blue-breast in all unvaccinated does, and the majority of these animals had to be culled soon after infection because of the severity of their condition. Strain CN.6708 produced classical blue-breast in only one of five unvaccinated does. The remainder presented with the abscess form.

Immunization of animals with alpha-toxoid had a dramatic effect on the clinical course of the infection produced by BB and Compton 201 strains. None of the five alpha-toxoid-immunized does challenged with strain BB developed blue-breast; all developed abscesses (Fig. 6a). Only one of four does challenged with strain Compton 201 developed blue-breast (Fig. 6b), which appeared only after 5 days. The other three animals developed abscesses. All five alpha-toxoid-immunized does challenged with strain CN.6708 developed abscesses (Fig. 6c).

Immunization with beta-toxin had no significant effect on infection by any of the three strains. Thus, four of five beta-toxin-immunized rabbits contracted blue-breast when challenged with strain Compton 201 (experiment 2, Fig. 7), compared with five of five nonvaccinates. A similar result was obtained with strain BB as the challenge strain. Thus, four of five beta-toxin-immunized does contracted blue-breast when challenged with strain BB compared with four of four controls (Fig. 6a). All five beta-toxin-immunized does challenged with strain CN.6708 developed abscesses (Fig. 6c). In experiment 1 (Fig. 6a), the animal with the highest anti-beta-toxin serum titer (no. 13) was the only one of the beta-immunized group not to contract blue-breast after BB challenge. Also, one animal (no. 44) in the beta-immunized group challenged with strain Compton 201 in experiment 2 (Fig. 7) did not contract blue-breast. In this case, however, the anti-beta level was no greater than that of two other animals (no. 43 and 45), which were not protected.

DISCUSSION

The role of extracellular toxins in staphylococcal disease has been the subject of many studies using a variety of animal models (14). Apart from the enterotoxins involved in food poisoning (6) and the epidermolytic toxin(s) produced in staphylococcal scalded skin syndrome (19), only alpha-toxin and possibly delta-toxin have been shown with any certainty to be produced by staphylococci in vivo and then only in localized necrotic lesions in small animals or in rodents dying from a large systemic challenge (16). Whether alpha-toxin, or indeed any other extracellular product of staphylococci, is of importance in those conditions where pus formation occurs remains uncertain.

The present study has shown that alpha-toxin plays a key role in the spreading hemorrhagic form of rabbit mastitis blue-breast seen in natural outbreaks and reproduced in the laboratory with two unrelated staphylococcal strains. A high circulating anti-alpha-toxin titer conferred protection against this lethal form of mastitis but only modified the clinical picture to the less severe abscess condition.

No significant protection against blue-breast or abscesses was seen in animals with high

TABLE 1. *Antibody levels in serum and milk produced by beta-toxin immunization*

Rabbit no.	Anti-beta titer (IU/ml)	
	Serum	Milk
1	40–80	2–4
2	8–16	1–2
3	20–40	2–4
4	40–80	2–4
5	10–20	2–4

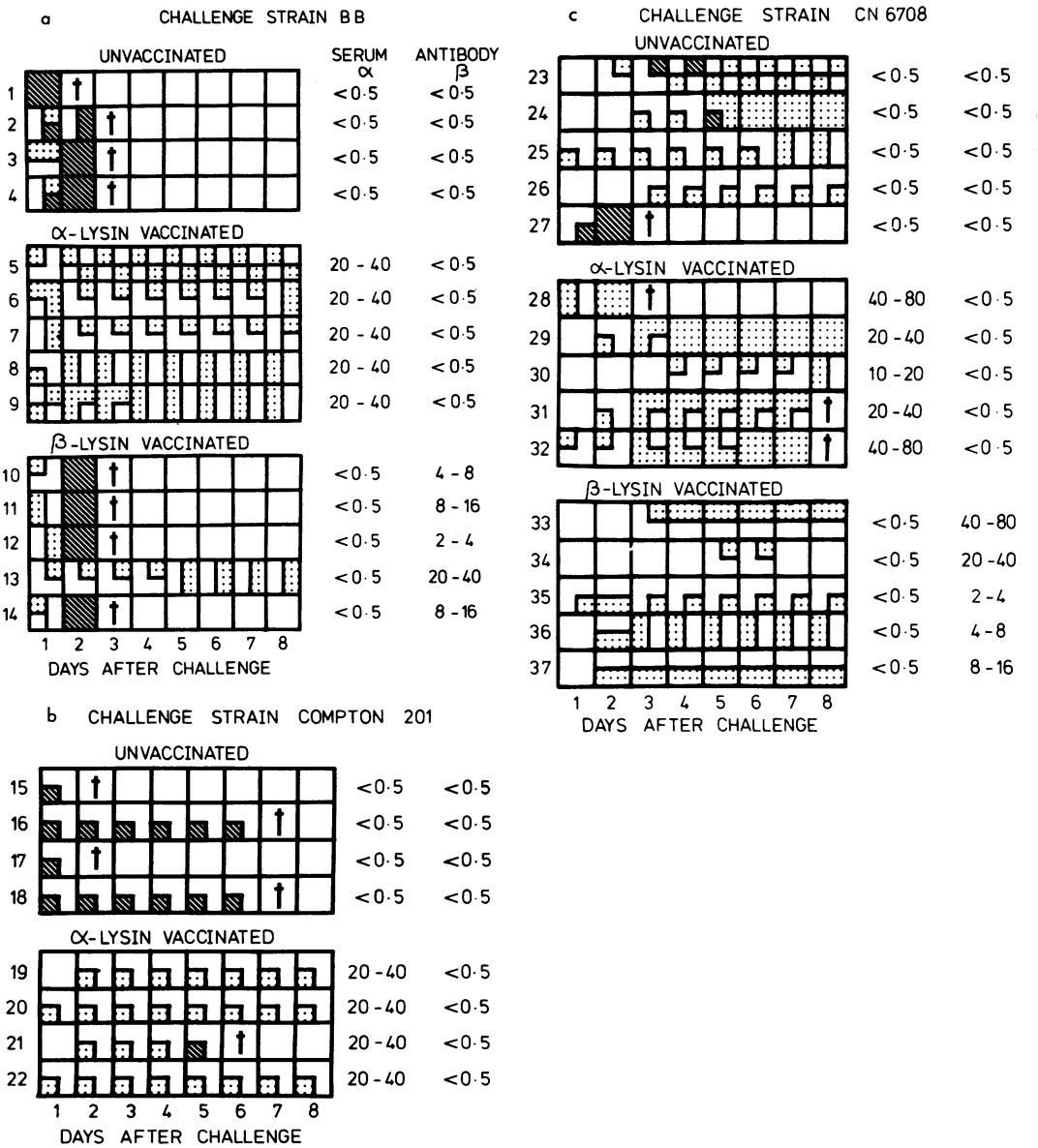

FIG. 6. *Results of infection experiment 1. Animals were observed for 8 days after infection. Each square represents the observations on a given day and is divided into quarters to represent the four challenged teats. Symbols:* ⁝⁝, *induration or lumpy-type lesion;* ▨, *blue-black spreading lesion;* †, *animal culled because of severity of lesions. Antibody levels are given in international units.*

circulating anti-beta-toxin titers, even though antibody was shown to be present in milk. The occasional animal appeared to be protected by immunization with this antigen against blue-breast, but this could not be attributed to antibody titer. Thus, the role of beta-toxin, a highly specific phospholipase (sphingomyelinase C) (13), in this or any other staphylococcal disease remains to be elucidated.

It is known that some staphylococcal strains (e.g., CN.6708) produce only abscesses in natural outbreaks of rabbit mastitis (3). In the bovine also, chronic mastitis typified by a high milk cell count is more common than the gangrenous form (4). Since neither of the antigens used in the present study protected against the abscess disease, further studies are needed to determine whether immunization with other

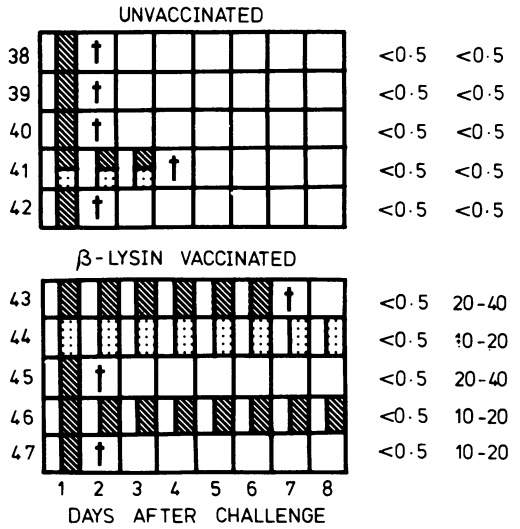

FIG. 7. *Results of infection experiment 2, using strain Compton 201 as the challenge strain. Legend and symbols as for Fig. 6.*

staphylococcal antigens might afford protection against these more chronic conditions.

The results presented here accord with those of Derbyshire (11) and Derbyshire and Smith (12), who produced chronic and gangrenous mastitis in goats. Whereas a high circulating anti-alpha-toxin titer was produced by using a crude cell toxoid vaccine and this prevented gangrene in these animals, chronic mastitis with a high milk cell count was still observed.

The importance of polymorphonuclear leukocytes in maintaining a balance between chronic and gangrenous mastitis in the bovine has recently been demonstrated in cows by Schalm et al. (23). Using anti-polymorph serum, a chronic condition was converted to a gangrenous one. It would seem that if a staphylococcal strain is able to produce sufficient alpha-toxin to destroy polymorphs, the disease becomes gangrenous. In the presence of antibody, however, the destructive effect of alpha-toxin on these cells is negated and the abscess condition results.

ACKNOWLEDGMENTS

We thank M. Collins for care and breeding of the animals used in this study, and Wendy Pearce and Susan Cowburn for their excellent technical assistance. In addition, thanks are due to P. D. Walker for discussion and advice.

LITERATURE CITED

1. **Adams, C. E.** 1961. Artificial insemination in the rabbit. Technical Bull. no. 1. Commercial Rabbit Association, Cheltenham, Gloucestershire, England.
2. **Adlam, C., C. M. Thorley, P. D. Ward, and M. Collins.** 1976. Staphylococcal mastitis in rabbits: description of a natural outbreak of the disease and attempts to reproduce the disease in the laboratory, p. 761-771. *In* J. Jeljaszewicz (ed.), Proceedings of the Third International Symposium on Staphylococci and Staphylococcal Infections, Warsaw 1975. Gustav Fisher Verlag, New York.
3. **Adlam, C., C. M. Thorley, P. D. Ward, M. Collins, R. N. Lucken, and P. A. Knight.** 1976. Natural and experimental staphylococcal mastitis in rabbits. J. Comp. Pathol. 86:581-593.
4. **Anderson, J. C.** 1976. Mechanisms of staphylococcal virulence in relation to bovine mastitis. Br. Vet. J. 132:229-245.
5. **Arbuthnott, J. P., J. H. Freer, and B. Billcliffe.** 1973. Lipid induced polymerisation of staphylococcal alpha toxin. J. Gen. Microbiol. 75:309-319.
6. **Bergdoll, M. S.** 1972. The enterotoxins, p. 301-331. *In* J. O. Cohen (ed.), The staphylococci. Wiley-Interscience, New York.
7. **Bernheimer, A. W., and L. L. Schwartz.** 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. 30:455-468.
8. **Blount, W. P.** 1945. Diseases of the mammary glands, p. 140-141. *In* Rabbit ailments. A short-treatise on the domestic rabbit in health and disease. Fur and Feather, Bradford, England.
9. **Colbeck, J. C.** 1956. Studies in hospital infection. I. The importance of fomites in the spread of staphylococcal infections with particular reference to mattresses and washing facilities. Can. Med. J. 12:563-580.
10. **Cowan, S. T., and K. J. Steel.** 1965. Manual for the identification of medical bacteria. Cambridge University Press, Cambridge.
11. **Derbyshire, J. B.** 1961. Further immunological studies in experimental staphylococcal mastitis. J. Comp. Pathol. 71:146-158.
12. **Derbyshire, J. B., and G. S. Smith.** 1969. Immunisation against experimental staphylococcal mastitis in the goat by the intramammary infusion of cell-toxoid vaccine. Res. Vet. Sci. 10:559-564.
13. **Doery, H. M., B. G. Magnusson, I. Gulasekharam, and J. E. Pearson.** 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40:283-296.
14. **Elek, S. D.** 1959. *Staphylococcus pyogenes* and its relation to disease. E. & S. Livingstone Ltd., Edinburgh.
15. **Frazier, W. C.** 1926. A method for the detection of changes in gelatin due to bacteria. J. Infect. Dis. 39:302-309.
16. **Kapral, F. A.** 1974. *Staphylococcus aureus*: some host-parasite interactions. Ann. N.Y. Acad. Sci. 236:267-276.
17. **Lupanov, M. T.** 1953. Clinical picture, treatment and prophylaxis of infectious mastitis in rabbits. (In Russian) Karakulevod. Zverovod. 6:52-53.
18. **McNiven, A. C., P. Owen, and J. P. Arbuthnott.** 1972. Multiple forms of staphylococcal alpha-toxin. J. Med. Microbiol. 5:113-122.
19. **Mellish, M. E., L. A. Glasgow, M. D. Turner, and C. B. Lillibridge.** 1976. The staphylococcal epidermolytic toxin, its discovery and review of recent progress, p. 473-488. *In* J. Jeljaszewicz (ed.), Proceedings of the Third International Symposium on Staphylococci and Staphylococcal Infections, Warsaw 1975. Gustav Fisher Verlag, New York.
20. **Moussu, G.** 1923. Affections des mamelles, p. 231-234. *In* Les principals maladies des habitants de la basse-cour et leur traitment, 2nd ed. Libraire Agricole de la Maison Rustique, Paris.
21. **Relyveld, E. H.** 1973. Préparation de vaccins antitoxiques et antimicrobiens à l'aide du glutaraldéhyde. C. R. Acad. Sci. Paris 277:613-616.
22. **Sawin, P. B.** 1950. The rabbit, p. 172-173. *In* E. J.

Farris (ed.), The care and breeding of laboratory animals. Chapman and Hall, London.
23. **Schalm, O. W., J. Lasmanis, and N. C. Jain.** 1976. Conversion of chronic staphylococcal mastitis to acute gangrenous mastitis after neutropenia in blood and bone marrow produced by an equine anti-bovine leucocyte serum. Am. J. Vet. Res. 37:885–890.
24. **Scheidegger, J. J.** 1955. Une micro-méthode de l'immunoelectrophorèse. Int. Arch. Allergy 7:103–110.
25. **Sierra, G.** 1957. A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek; J. Microbiol. Serol. 23:15–22.
26. **Soituz, V.** 1930. Sur un staphylocoque adapté à la glande mammaire de la lapine. Rev. Pathol. Comp. Hyg. Gen. 30:381–387.
27. **Winkler, K. C., J. De Waart, C. Grootsen, B. J. M. Zegers, N. F. Tellier, and C. D. Vertregt.** 1965. Lysogenic conversion of staphylococci to loss of beta toxin. J. Gen. Microbiol. 39:321–333.