THE ACTION OF FORMALDEHYDE ON STAPHYLOCOCCAL δ HAEMOLYSIN

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PLATE XIV

IN CONTRAST to other staphylococcal haemolysins that have restricted species specificities, the δ haemolysin is cytolytic for a wide range of erythrocytes (Kreger *et al.*, 1971; Wiseman, 1975), cultured cells (Gladstone and Yoshida, 1967; Thelestam and Möllby, 1975), cellular organelles and bacterial protoplasts and sphaeroplasts (Kreger *et al.*, 1971). When administered to guinea-pig tissues, δ haemolysin inhibits water absorption and elevates cyclic AMP levels in the ileum and causes increased vascular permeability in the skin (Kapral *et al.*, 1976; O'Brien and Kapral, 1976). Delta-haemolysin also binds to phospholipids (Kapral, 1972) and to lipoproteins by the phospholipid moiety (Whitelaw and Birkbeck, 1978).

The properties of the molecule that cause cytolysis, increase cyclic-AMP levels, enable binding to phospholipids and induce an immune response are not known. We have investigated the action of formaldehyde on δ haemolysin in attempts to produce "toxoids" for immunisation of animals and to determine whether some of the biological properties of δ haemolysin are mediated by common mechanisms.

MATERIALS AND METHODS

Staphylococcal δ haemolysin. The properties, purification and assay of the δ haemolysin of *Staphylococcus aureus* strain NCTC10345 have been described by Chao and Birkbeck (1978*a* and *b*) and Birkbeck and Whitelaw (1980).

Reaction of δ haemolysin with formaldehyde. The method was based on that of Warren, Spero and Metzger (1973); formaldehyde (Analar Grade, BDH Chemicals Ltd, Poole, Dorset) was added to solutions of δ haemolysin (1 or 2 mg/ml) in buffers at pH 5 (0.15M phosphate), pH 7.5 (0.15M phosphate) or pH 9.5 (0.05M bicarbonate/carbonate) to a final concentration of 1% (w/v). After incubation for up to 7 days, the reaction mixtures were dialysed against 0.05M phosphate buffer at pH 6.8 to remove excess formaldehyde. The formalised preparations were centrifuged at 10 000 *g* for 20 min. to remove the insoluble fraction.

Analytical procedures. Immuno-electrophoresis, protein assays and sodium dodecyl sulphate (SDS) polyacrylamide-gel electrophoresis were carried out as described by Birkbeck and Whitelaw (1980). Isoelectric focusing was done by the method of Talbot and Caie (1975). Counter-immuno-electrophoresis was performed in 1% agarose (Koch-Light Ltd, Colnbrook, Bucks) 2 mm deep on 75 × 75-mm glass plates by the method of Culliford (1964) to detect antibodies to δ haemolysin in sera; in the conditions used, α and β lipoproteins did not

interfere with detection of antibody because of their acidic isoelectric points (pH 5.5 and pH 5.4 respectively; Young, 1962).

Inhibition of δ haemolysin by lecithin. Chromatographically pure ovoid lecithin was a gift from Dr J. H. Freer. Solutions of lecithin (0.5 mg in 0.5 ml of chloroform) were evaporated to dryness under a stream of nitrogen in the bottom of 100×12.5 -mm test tubes and 0.2 ml of phosphate-buffered saline (PBS; Dulbecco and Vogt, 1954) was added. The mixture was resuspended and, after equilibration for 1 h, sonicated briefly in a 125W sonic cleaning bath (Polaron, Watford, Herts). Delta haemolysin or formaldehyde-treated δ haemolysin (125 μ g in 0.2 ml) was added and after 45 min. at 37°C each solution was tested by immunodiffusion to detect unbound antigen.

Binding of δ haemolysin to erythrocytes. Delta haemolysin or formaldehyde-treated δ haemolysin (50 μ g in 0.1 ml) was mixed with 0.7 ml of 50% washed cod erythrocytes. After incubation for 30 min. at 20°C, erythrocytes and bound protein were removed by centrifugation for 5 min. at 500 g and the supernatant fluid was tested for unbound antigen by immunodiffusion.

Immunisation of animals. HAM/1CR mice (4 weeks old) were given intraperitoneal injections of 0.05 ml of a mixture that contained 50 μ g of δ haemolysin in either Freund's complete adjuvant (Bayol F, 0.6 ml; Arlacel A, 0.2 ml; δ haemolysin solution, 0.2 ml; *Mycobacterium tuberculosis* 80 μ g; Stewart-Tull and White, 1967) or in PBS. The number and timing of injections are given in the *Results* section. Blood was obtained by cardiac puncture during terminal ether anaesthesia and serum samples were stored at -20°C until required.

One rabbit was immunised with δ haemolysin treated with formaldehyde for 7 days at pH 5. After three intramuscular injections of the preparation that contained 100 μ g of protein in Freund's complete adjuvant on days 1, 3 and 5 and intravenous injections that contained 100 μ g of protein in PBS on days 40, 43 and 45, blood was obtained from the marginal ear vein on day 52 and the serum was separated. The preparation of rabbit antiserum to native δ haemolysin has been described by Birkbeck and Whitelaw (1980). Antisera were absorbed by mixing equal volumes of serum and a 1 mg/ml solution of δ haemolysin.

Measurement of increased vascular permeability in rabbit skin. The backs of New Zealand white rabbits were shaved and 0.1-ml volumes of δ haemolysin preparations were injected intradermally. Each test sample was injected in triplicate into each of three rabbits and standard δ haemolysin preparations (62.5–1000 μ g) were injected into eight sites on each rabbit. After 1 h, 3 ml of 5% Pontamine Sky Blue dye in physiological saline was injected and 1 h later the diameter of the blue zone around each injection site was measured with calipers.

RESULTS

Immunisation of mice with δ haemolysin

In a series of experiments, groups of five mice were immunised with a preparation that contained 50 μ g of haemolysin in Freund's complete adjuvant. Either one primary injection or three injections on days 1, 3 and 5 were followed by either one booster injection or three injections given at 2-day intervals; the booster injections contained 50 μ g of δ haemolysin in saline and were given 1, 2, 3 or 5 weeks after the primary injection. Sera obtained 1 week after the final injection were pooled and tested for anti- δ -haemolysin antibody by counter-immunoelectrophoresis. Anti- δ haemolysin was detected only in pooled serum from mice that received three primary injections and three booster injections after an interval of 5 weeks. However, in subsequent experiments the quantity of antigen was varied from 6.25 to 100 μ g per injection and the *M. tuberculosis* concentration in the Freund's complete

adjuvant was varied from 1.25 to 5 $\mu\text{g}/0.05$ ml, but antibody was not detected in any of the 15 serum pools. Individual sera were therefore used in later experiments so that the proportion of mice responding to each immunogen could be determined.

Effect of formaldehyde treatment on δ haemolysin

The haemolysin was chemically modified by treatment with formaldehyde at pH 5, pH 7.5 or pH 9.5. Haemolytic activity was lost rapidly during treatment at pH 5 or pH 7.5 (fig. 1) but antigenic reactivity detected by counter-immunoelectrophoresis and gel diffusion with anti- δ -haemolysin serum was retained even after treatment with formaldehyde for 7 days at pH 5, 7.5 or 9.5. In a series of experiments mice were immunised with formaldehyde-treated δ haemolysin and the resulting sera were analysed individually for anti- δ -haemolysin antibodies. The proportion of mice responding to the δ haemolysin preparations increased with the number of injections of antigen and also with the interval between primary and booster injections (table I). Of the 80 mice immunised with each preparation, 23% responded to native δ haemolysin or δ haemolysin treated with formaldehyde at pH 9.5; 39% responded to δ haemolysin treated at pH 7.5, and 61% to δ haemolysin treated at pH 5. This enhanced response to the formaldehyde-treated δ -haemolysin preparations was found only in samples incubated with formaldehyde for 7 days at pH 5 or 7.5. Delta-haemolysin treated with formaldehyde for 7 days at pH 5 or 7.5 lost all haemolytic activity but still induced increased vascular

FIG. 1.—Loss of haemolytic activity of δ haemolysin incubated with formaldehyde at 37°C, at pH 5 (●—●), pH 7.5 (▲—▲), or pH 9.5 (■—■). Delta haemolysin was also incubated at pH 5 (○—○), pH 7.5 (△—△), and pH 9.5 (□—□) without formaldehyde.

TABLE I
Immunogenicity of native and formaldehyde-treated δ haemolysin in mice

Timing (days) of injections		Percentage of animals in each group of 9–12 mice with serum antibody detected after immunisation with			
in FCA	in saline	untreated δ haemolysin	δ haemolysin treated with formaldehyde at		
			pH 5	pH 7.5	pH 9.5
1	22	0	45	0	10
1	22,24,26	20	22	30	25
1	36	20	67	20	0
1	36,38,40	40	89	70	30
1,3,5	22	0	20	30	10
1,3,5	22,24,26	30	80	54	56
1,3,5	36	20	75	20	10
1,3,5	36,38,40	50	100	90	50

FCA = Freund's complete adjuvant.

permeability in rabbit skin although the effect was less than with native δ haemolysin (table II).

Physico-chemical properties of formaldehyde-treated δ haemolysin

The treatment of δ haemolysin with formaldehyde resulted in an increased electrophoretic mobility at pH 8.6 (fig. 2) with a concomitant decrease in isoelectric point (fig. 3). Treatment with formaldehyde at pH 5 for 7 days resulted in precipitation of approximately 55% of the protein in the preparation but soluble polymers of the basic subunit of δ haemolysin were not

TABLE II
Properties of δ haemolysin treated with formaldehyde for 7 days at pH 5, 7.5 or 9.5

Property	Activity of			
	δ haemolysin preparation treated with formaldehyde at			native δ haemolysin
	pH 5	pH 7.5	pH 9.5	
Haemolytic activity	<0.1%*	<0.1%*	3%*	100%*
Vascular permeability induced in rabbit skin	36%*	56%*	74%*	100%*
Immune response induced in mice	61%†	39%†	23%†	23%†
Reaction with antibody in gel diffusion	+	+	+	+
Binding to lecithin liposomes	—	—	—	+
Isoelectric point	pH 4.5	pH 4.1	pH 4.2	pH 4.9

* = Percentage reactivity; native δ haemolysin = 100%.

† = Percentage of animals that gave an immune response to any dosage regimen with the given preparation.

FIG. 3.—Isoelectric focusing of native δ haemolysin (a) and δ haemolysin treated with formaldehyde for 7 days at pH 5 (b), pH 7.5 (c) and pH 9.5 (d)., pH; —●—●—●, E_{280} .

detected by SDS-polyacrylamide-gel electrophoresis of the preparations treated at pH 5, 7.5 or 9.5.

Lecithin did not significantly inhibit the precipitinogenic activity of formaldehyde-treated δ haemolysins at a concentration that inhibited the lytic and precipitinogenic activity of native δ haemolysin (fig. 4). Similarly, formaldehyde-treated δ haemolysin, but not native δ haemolysin, was detected by immunodiffusion after incubation with cod erythrocytes; this indicated that treatment with formaldehyde destroyed the ability of δ haemolysin to bind to erythrocytes.

Properties of antisera to formaldehyde-treated δ haemolysin

Antisera raised in mice to formaldehyde-treated δ haemolysin reacted with native and with formaldehyde-treated δ haemolysin in counter-immunoelectrophoresis, but gave a pronounced precipitin line by immunodiffusion only with formaldehyde-treated δ haemolysin. Absorption of the antisera with native δ haemolysin did not remove all precipitinogenic activity against formaldehyde-treated δ haemolysin.

Rabbit antiserum to δ haemolysin treated with formaldehyde at pH 5 reacted more strongly in immunodiffusion with native δ haemolysin than mouse antisera and also contained antibodies to formaldehyde-treated δ haemolysin which were not removed by absorption with native δ haemolysin (fig. 5).

DISCUSSION

Native δ haemolysin was poorly immunogenic in the mouse; it induced a response in, at most, 50% of immunised animals (table I) whereas in the rabbit an immune response was obtained in all animals tested (Birkbeck and Whitelaw, 1980). The poor response to multiple injections with Freund's adjuvant and a range of antigen doses indicates that the poor response was not due to an inadequate immunisation schedule.

Formaldehyde treatment affected several properties of δ haemolysin. The rapid loss of haemolytic activity at pH 5 or 7.5 with retention of antigenic reactivity were similar to a classical 'toxoiding' reaction, and immunogenicity was markedly enhanced. Enhanced immunogenicity has been described after chemical modification of gelatin (Sela and Arnon, 1960), staphylococcal enterotoxin B (Warren *et al.*, 1973) and staphylococcal exfoliatin A and B (Kondo, Sakurai and Sarai, 1976). Warren *et al.* (1973) considered that treatment with formaldehyde at neutral or acid pH caused polymerisation of the enterotoxin-B molecules and that this was responsible for the enhanced immunogenicity. This is unlikely to be the predominant factor in the present case because oligomers of δ haemolysin were not detected by SDS-polyacrylamide-gel electrophoresis in the soluble fraction used for immunisation of mice. The action of formaldehyde on protein toxins has been discussed by Blass, Bizzini and Raynaud (1967) and Bizzini and Raynaud (1974). The initial interaction of formaldehyde with free amino groups may be followed by the formation of intermolecular or intramolecular methylene bridges with other free amino groups or with tryptophan, tyrosine or histidine residues. Because tyrosine and histidine were absent from the δ haemolysin (Birkbeck and Whitelaw, 1980) molecular changes were probably restricted to intramolecular methylene bridge formation with tryptophan or lysine residues.

Kondo *et al.* (1976) found that whereas native staphylococcal exfoliatin A and B were not immunogenic in the mouse, diazotisation or nitration rendered both proteins immunogenic. The failure of Kondo *et al.* (1976) to enhance immunogenicity by treatment with formaldehyde at pH 8.8 agrees with the

finding in the present study and in that of Warren *et al.* (1973) that immunogenicity was not enhanced by treatment with formaldehyde in alkaline conditions; moreover, antigenic reactivity may be significantly depressed due to greater modification of antigenic determinants at alkaline pH (Warren *et al.*, 1973).

The reaction of surface free amino groups with formaldehyde could lead to a reduced isoelectric point in δ haemolysin (fig. 2) but this is unlikely to affect immunogenicity of the protein. The enhanced immunogenicity is more probably a consequence of the reduced affinity of δ haemolysin for lecithin (fig. 3) because, during immunisation, this would leave formaldehyde-treated δ haemolysin free to stimulate an immune response rather than being bound to lipoproteins or cell-membrane phospholipids. However, all formaldehyde-treated preparations had reduced affinity for lecithin liposomes, and other factors such as the minimal change in antigenic determinants induced by formaldehyde at acid or neutral pH when compared with the changes at alkaline pH (Warren *et al.*, 1973), may also be important. The loss of affinity of formaldehyde-treated haemolysin for erythrocytes and phospholipids, with consequent loss of haemolytic activity, may indicate that free amino groups are important in the initial interaction with the cell membrane and liposomes. It is noteworthy that in the amphipathic polypeptide, bee melittin, which shares many properties with δ haemolysin (Freer and Arbuthnott, 1976) the initial binding to membranes is thought to be mediated by ionic interactions followed by post-binding conformational changes resulting in membrane penetration (Dawson *et al.*, 1978).

Although haemolytic activity was rapidly reduced by treatment with formaldehyde, increased vascular permeability in rabbit skin was affected only slightly. We have recently shown (Stearne and Birkbeck, 1979) that increased vascular permeability can be mediated by tryptic fragments of the molecule that are devoid of detectable haemolytic activity or antigenic reactivity. The biological activities of the molecule are therefore not mediated by a common mechanism and further characterisation of the molecule is required to establish the basis for the activities of δ haemolysin.

SUMMARY

Native staphylococcal δ haemolysin was poorly immunogenic in the mouse. After treatment with formaldehyde at pH 5 or pH 7.5, haemolytic activity was rapidly lost but antigenic reactivity retained and, at pH 5, immunogenicity was enhanced. Treatment of the haemolysin with formaldehyde at pH 9.5 reduced haemolytic activity by 97% but did not enhance immunogenicity. There was no evidence of polymerisation of formaldehyde-treated δ haemolysin, and the enhanced immunogenicity was considered to be partly due to a reduced affinity for phospholipids.

REFERENCES

- BIRKBECK, T. H. AND WHITELAW, D. D. 1980. Immunogenicity and molecular characterisation of staphylococcal delta-haemolysin. *J. med. Microbiol.*, **13**, 213.
- BIZZINI, B. AND RAYNAUD, M. 1974. La détoxification des toxines protéiques par le formol: mécanismes supposés et nouveaux développements. *Biochimie*, **56**, 297.
- BLOSS, J., BIZZINI, B. AND RAYNAUD, M. 1967. Études sur le mécanisme de la détoxification des toxines protéiques par le formol. *Bull. Soc. Chim. biol., Paris*, 3957.
- CHAO, L.-P. AND BIRKBECK, T. H. 1978a. Assay of staphylococcal δ haemolysin with fish erythrocytes. *J. med. Microbiol.*, **11**, 303.
- CHAO, L.-P. AND BIRKBECK, T. H. 1978b. Storage of marine fish erythrocytes in liquid nitrogen. *J. fish Biol.*, **13**, 483.
- CULLIFORD, B. J. 1964. Precipitin reactions in forensic problems. *Nature, Lond.*, **201**, 1092.
- DAWSON, C. R., DRAKE, A. F., HELLIWELL, J. AND HIDER, R. C. 1978. The interaction of bee melittin with lipid bilayer membranes. *Biochim. biophys. Acta*, **510**, 75.
- DULBECCO, R. AND VOGT, M. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. exp. Med.*, **99**, 167.
- FREER, J. H. AND ARBUTHNOTT, J. P. 1976. Biochemical and morphological alterations of membranes by bacterial toxins. In *Mechanisms in bacterial toxinology*, edited by A. W. Bernheimer. Wiley: New York, p. 169.
- GLADSTONE, G. P. AND YOSHIDA, A. 1967. The cytopathic action of purified staphylococcal δ haemolysin. *Br. J. exp. Path.*, **48**, 11.
- KAPRAL, F. A. 1972. Inhibition of *Staphylococcus aureus* delta-hemolysin by phospholipids. *Proc. Soc. exp. Biol. Med.*, **141**, 519.
- KAPRAL, F. A., O'BRIEN, A. D., RUFF, P. D. AND DRUGEN, W. J. 1976. Inhibition of water absorption in the intestine by *Staphylococcus aureus* delta-toxin. *Infect. Immun.*, **13**, 140.
- KONDO, I., SAKURAI, S. AND SARAI, Y. 1976. Staphylococcal exfoliatin A and B. In *Staphylococci and staphylococcal diseases*, edited by J. Jeljaszewicz. Fischer: Stuttgart, p. 489.
- KREGER, A. S., KIM, K.-S., ZABORETZKY, F. AND BERNHEIMER, A. W. 1971. Purification and properties of staphylococcal delta-hemolysin. *Infect. Immun.*, **3**, 449.
- O'BRIEN, A. D. AND KAPRAL, F. A. 1976. Increased cyclic adenosine 3',5'-monophosphate content in guinea pig ileum after exposure to *Staphylococcus aureus* delta-toxin. *Infect. Immun.*, **13**, 152.
- SELA, M. AND ARNON, R. 1960. Studies on the chemical basis of the antigenicity of proteins. 1. Antigenicity of polypeptidyl gelatins. *Biochem. J.*, **75**, 91.
- STEARNE, L. E. T. AND BIRKBECK, T. H. 1979. Vascular permeability changes in rabbit skin induced by staphylococcal δ -lysin. *Toxicon*, **17**, suppl. 1, 177.
- STEWART-TULL, D. E. S. AND WHITE, R. G. 1967. The influence of age of culture on the production of adjuvant-active peptidoglycolipids by saprophytic bacteria. *Immunology*, **12**, 349.
- TALBOT, P. AND CAIE, I. S. 1975. A horizontal trough apparatus for isoelectric focusing. In *Isoelectric focusing*, edited by J. P. Arbuthnott and J. A. Beeley. Butterworth: London, p. 74.
- THELESTAM, M. AND MÖLLBY, R. 1975. Determination of toxin-induced leakage of different-size nucleotides through the plasma membrane of human diploid fibroblasts. *Infect. Immun.*, **11**, 640.
- WARREN, J. R., SPERO, L. AND METZGER, J. F. 1973. Antigenicity of formaldehyde-inactivated staphylococcal enterotoxin B. *J. Immunol.*, **111**, 885.
- WHITELAW, D. D. AND BIRKBECK, T. H. 1978. Inhibition of staphylococcal delta-haemolysin by human serum lipoproteins. *FEMS microbiol. Lett.*, **3**, 335.
- WISEMAN, G. M. 1975. The hemolysins of *Staphylococcus aureus*. *Bact. Rev.*, **39**, 317.
- YOUNG, E. G. 1962. In *Comprehensive biochemistry*, edited by M. Florkin and E. H. Stotz. Elsevier: Amsterdam, vol. 7, p. 22.

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FIG. 2.—Immuno-electrophoresis of formaldehyde-treated δ haemolysin. D= δ haemolysin; D5, D75, D95= δ haemolysin treated with formaldehyde at pH 5, pH 7.5 and pH 9.5 respectively; all troughs were filled with rabbit antiserum to δ haemolysin.

FIG. 4.—Immunodiffusion analysis of a mixture of lecithin and δ haemolysin with antiserum to δ haemolysin. 1=rabbit anti- δ haemolysin; 2, 3, 4= δ haemolysin treated with formaldehyde at 37°C for 7 days at pH 9.5, pH 7.5 or pH 5 respectively and then mixed with lecithin; 5= native δ haemolysin with lecithin; 6= δ haemolysin alone; 7, 8 and 9= δ haemolysin incubated at 37°C for 7 days at pH 9.5, pH 7.5 or pH 5 respectively and then mixed with lecithin.

FIG. 5.—Immunodiffusion analysis of rabbit antiserum to δ haemolysin treated with formaldehyde at pH 5 absorbed with native δ haemolysin. 1=rabbit antiserum to δ haemolysin treated with formaldehyde for 7 days at pH 5 and absorbed with native δ haemolysin, 62.5 μ g/ml; 2= δ haemolysin, 62.5 μ g/ml; 3, 4 and 5= δ haemolysin treated with formaldehyde for 7 days at pH 5 (3), pH 7.5 (4) or pH 9.5 (5); 6, 7 and 8= δ haemolysin incubated for 7 days at pH 5 (6), pH 7.5 (7) or pH 9.5 (8).

[facing page 230.]