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# STAPHYLOCOCCAL DELTA-HEMOLYSIN INHIBITS CELLULAR BINDING OF EPIDERMAL GROWTH FACTOR AND INDUCES ARACHIDONIC ACID RELEASE

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SUMMARY: Staphylococcal delta-hemolysin inhibited the binding of epidermal growth factor to cell surface receptors in rat embryo and HeLa cells at concentrations of 1 - 10 μg/ml. At these concentrations, delta-hemolysin was not cytolytic. Delta-hemolysin also induced arachidonic acid release and prostaglandi synthesis in 10T½ cells. Thus, this polypeptide shares several effects in cell culture with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate.

Recent studies indicate that the potent tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) at nanomolar concentrations specifically inhibits the binding of epidermal growth factor (EGF) to its cell surface receptors (1,2). TPA exerts a number of other effects on membrane structure and function and there is a considerable body of data suggesting that cell membranes may be the primary site of action of this and related compounds. (For review see refs. 1,2,3). It is of interest, therefore, that recent studies indicate that the bee venom polypeptide melittin, which is known to insert into the lipid bilayer of membranes and to thereby perturb membrane structure, induces several effects on cell cultures that simulate those produced by TPA (4).

Staphylococcus aureus produces four hemolysins as major exotoxins. One of these, delta-hemolysin, lyses rabbit, sheep, horse and human erythrocytes (5,6) and also disrupts leukocytes and tissue culture cells, when used at relatively high concentrations (7). Recent amino acid sequence studies indicate that the purified material has a molecular weight of 2977 and consists of a 26 amino acid polypeptide chain which contains no disulfide bridges (8). The present paper provides evidence that like TPA, delta-hemolysin is a potent inhibitor of EGF-receptor binding. Also like TPA (4,9,10) and melittin (4), it induces the release from membrane phospholipids of arachidonic acid and stimulates prostaglandin synthesis. It may be useful, therefore, to produce specific perturbations in membrane function and to assess the relevance of these to the process of tumor promotion.

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## Materials and Methods:

Materials: A cloned fibroblastic cell line (FRE-8D) prepared from rat embryos was provided by Dr. P. Fisher of our Institute. EGF and $^{125}$I-EGF (specific activity, 120 mCi/mg) were purchased from Collaborative Research Inc. [5,6,8,9, 11,12,14,15-$^{3}$H]-Arachidonic acid (specific activity, 84 Ci/mmol) was obtained from Amersham. Arachidonic acid and prostaglandins E2 and F2$\alpha$ were purchased from Sigma Chemical Co. Delta-hemolysin was isolated and purified by the method of Heatley (11

Methods: The EGF-cell surface binding assay was carried out essentially as described previously (2). The FRE-8D cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The cell monolayer was washed with phosphate buffered saline (PBS). Two ml of serum-free DMEM containing 1 mg/ml bovine serum albumin and 0.05 $\mu$Ci of $^{125}$I-EGF were added. Delta-hemolysin was added just prior to the addition of $^{125}$I-EGF. The plates were incubated for the times indicated in the legends at 37$^{0}$C in a 5% $CO_2$ atmosphere. Then the medium was removed, the cells were washed 3 times with cold DMEM and solubilized with 1 ml of 1% Triton X-100; 5 mg/ml trypsin; 2 mg/ml EDTA. After an hour incubation, the cell lysate was removed and 0.5 ml of 1% sodium dodecyl sulfate was added. The radioactivity of the total lysate was counted by liquid scintillation. The value of nonspecific binding (100-200 cpm) was substracted from all binding assay data. HeLa cells were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum. EGF binding was carried out in the same way as with FRE-8D cells. Arachidonic acid and prostaglandin release were assayed as described previously with 10T½ cells (4,5). The cytolytic effect of delta-hemolysin was observed microscopically. Confluent or subconfluent cells were incubated in serum-free DMEM. Morphological changes were monitored by phase contrast microscopy (7).

Results: Under our experimental conditions, EGF binding was saturated after 2 hours in FRE-8D cells. The addition of 2.5 $\mu$g/ml of delta-hemolysin at time

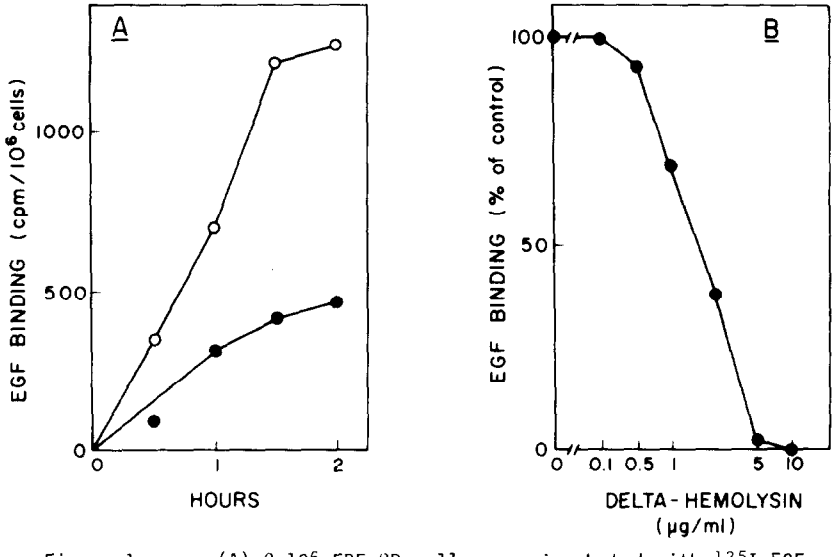


Figure 1: (A) 2x10$^{6}$ FRE-8D cells were incubated with $^{125}$I-EGF (0.05 $\mu$Ci, 0.2 ng) with (●) or without (○) 2.5 $\mu$g/ml of delta-hemolysin. (B) The cells were incubated with $^{125}$I-EGF and the indicated concentrations of delta-hemolysin for 2 hours. Values are means of duplicate samples.

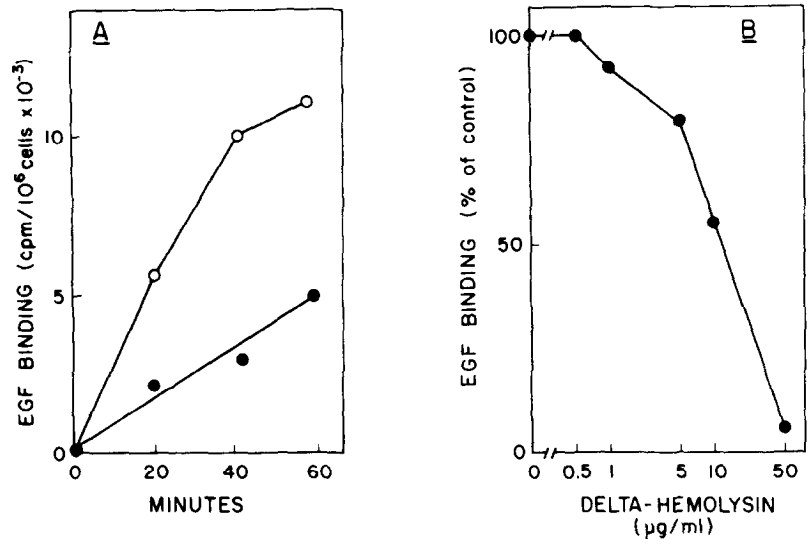


Figure 2: (A) $2x10^6$ HeLa cells were incubated with $^{125}I$-EGF with (●) or without (○) 10 µg/ml of delta-hemolysin. (B) The cells were incubated with $^{125}I$-EGF and the indicated concentrations of delta-hemolysin for 60 minutes. Values are means of duplicate samples.

zero resulted in a rapid and marked inhibition of EGF binding, as shown in Figure 1A. The 50% dose required to produce 50% inhibition was about 2 µg/ml and complete inhibition was obtained with about 5 µg/ml (Figure 1B). Parallel studies indicated that no cytolytic effect was observed at concentrations as high as 10 µg/ml of delta-hemolysin, after incubation for 2 hours at 37°C, although higher concentrations were toxic.

Delta-hemolysin also inhibited EGF binding in HeLa cells (Figure 2A). With these cells 50% inhibition was observed at about 10 µg/ml (Figure 2B) and again no cytolytic effect was observed at this concentration of delta-hemolysin. HeLa cells displayed slight swelling at 50 µg/ml of delta-hemolysin after a 1 hour incubation. Inhibition of EGF binding by delta-hemolysin was not affected by 50 µg/ml of hydrocortisone or dexamethasone (12) or 25 µg/ml of mepacrine (13), agents reported to inhibit membrane phospholipases.

Studies on the release of arachidonic acid were done with the 10T½ mouse embryo cells to compare the effects with those previously reported for TPA and melittin (4,9). 10T½ cells were more sensitive to the cytolytic effect of delta-hemolysin than FRE-8D or HeLa cells. The cells began to swell at 5 µg/ml of delta-hemolysin after 1 hour incubation. At 1 µg/ml, however, no cytolysis was observed even at 4 hours. At 1 µg/ml delta-hemolysin proved to be a potent inducer of the release of arachidonic acid metabolites from the

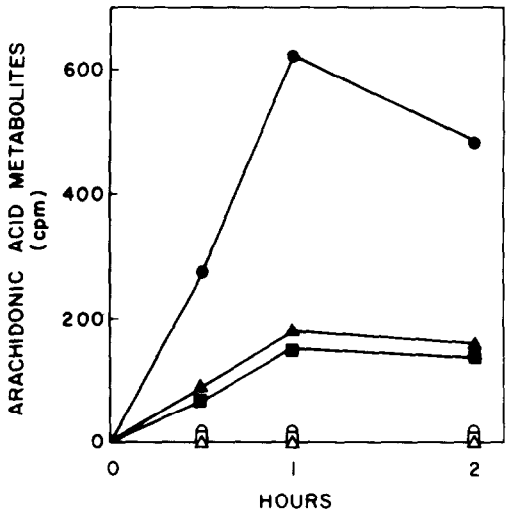


Figure 3: 10T½ cells prelabelled with $^3$H-arachadonic acid were incubated in 2ml of serum-free DMEM with (black symbols) or without (white symbols) 1 μg/ml of delta-hemolysin. At the indicated times, 1.8ml aliquots of media were extracted with ethyl acetate, and after evaporation, 0.1ml of chloroform was added and 20 μl was analyzed by silica gel thin-layer chromatography. The spots corresponding to arachidonic acid (●,○), prostaglandin $E_2$ (▲,△) and prostaglandin $F_{2\alpha}$ (■,□) were removed and counted. Values are means of duplicate samples.

$^3$H-arachidonic acid prelabelled cells (Figure 3). The release of metabolites into the medium was apparent within 30 minutes and reached a maximum at 1 hour. Chromatographic analysis of the media showed that free arachidonic acid and significant amounts of prostaglandins E2 and F2α were released in cells exposed to 1 μg/ml of delta-hemolysin (Figure 3).

Disscussion: The present studies indicate that a concentrations of 1 - 10 μg/ml delta-hemolysin causes a rapid inhibition of EGF binding to cell surface receptors in a rat embryo fibroblast line and in HeLa cells. On a molar basis, this compound is roughly equivalent to TPA in terms of potency for this effect. The effect occurred without evidence of gross disruption of the cell surface membrane. It is known that although the initial binding is rapid, there is a lag of about 30 min. before the EGF-receptor complex is internalized (14,15). Since the binding of EGF was inhibited almost immediately after adding delta-hemolysin is unlikely that it acts by enhancing internalization and degradation of EGF. Previous results suggest that inhibition by TPA of EGF binding is due to alterations in the conformation of EGF receptors in cell membranes, perhaps related to changes in the lipid microenvironment (2). Similar mechanisms might explain the action of delta-hemolysin. Presumably, the ability of TPA, melittin and delta-hemolysin to induce arachidonic acid release reflects stimulation of

phospholipid deacylation due to activation of membrane bound enzymes as a consequence of perturbations in membrane structure produced by these compounds. These data do not of course, indicate that the primary site of binding of these compounds is the same. Both TPA and delta-hemolysin stimulate lymphocyte mitosis (16,17) and both induce epidermal inflammation (5,18). It will be of interest to determine to what extent delta-hemolsyin shares other effects with tumor promoters.

References:

1. Lee, L.S. and Weinstein, I.B. (1978) Science 202, 313-315.
2. Lee, L.S. and Weinstein, I.B. (1979) Proc. Natl. Acad. Sci. USA, 76, 5168-5172.
3. Weinstein, I.B., Yamasaki, H., Wigler, M., Lee, L.S., Fisher, P.B., Jeffrey, A.M. and Grunberger, D. (1979) in Carcinogens: Identification and Mechanisms of Action, (A.C. Griffin and C.R. Shaw, eds.), Raven Press, New York, pp. 399-418.
4. Mufson, R.A., Laskin, J.D., Fisher, P.B. and Weinstein, I.B. (1979) Nature 280, 72-74.
5. Marks, J. and Vaughan, A.C.T. (1950) J. Path. Bact., 62, 597-615.
6. Kreger, A.S., Kim, K., Zaboretzky, F. and Bernheimer, A.W. (1971) Infection and Immunity, 3, 449-465.
7. Gladstone, G.P. and Yoshida, A. (1967) Brit. J. Exp. Path., 48, 11-19.
8. Fitton, J.E. and Shaw W.V., manuscript in preparation.
9. Mufson, R.A., Defeo,D. and Weinstein, I.B. (1979) Mol. Pharmacol., 16, 569-578.
10. Levine, L. and Hassid, A. (1977), Biochem, Biophys. Res. Comm. 79, 477-483
11. Heatley, N.G. (1971) J. Gen. Microbiol., 69, 269-278.
12. Lewis, G.P. and Piper, P.J. (1978) Biochem. Pharm., 27, 1409-1412.
13. Vargaftig, B.B. and Hai, N.D. (1972) J. Pharm. Pharmacol., 24, 159-161.
14. Carpenter, G. and Cohen, S. (1979) Ann. Rev. Biochem., 48, 193-216.
15. Haigler, H., Ash, J.F., Singer, S.J. and Cohen, S. (1978) Proc. Natl. Acad. Sci. USA, 75, 3317-3321.
16. Kensler, T.W. and Mueller, G.C. (1978) Cancer Res. 38, 771-775.
17. Evans, L. and Lack, C.H. (1969) Life Sciences 8, 677-681.
18. Slaga, T.J., Sivak, A. and Boutwell, R.K. (eds.), (1978) Carcinogenesis, Vol. 2. Mechanisms of Tumor Promotion and Cocarcinogenesis, Raven Press, New York.