Differences in Binding of the Direct Lytic Factor (DLF) of Cobra Venom (Naja naja) to Intact Red Cells and Ghosts

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Summary. The binding of direct lytic factor (DLF) from cobra venom (Naja naja) to intact guinea-pig red cells and to guinea-pig ghosts was estimated quantitatively by bioassay of DLF in the supernatant.

1. DLF was not bound to intact red cells in considerable amounts, during 320 min incubation.
2. The degree of binding to ghosts was much larger than that in suspensions of intact red cells. Binding to ghosts increased with time.
3. Whereas the binding of DLF to ghosts was not much influenced by varying the incubation temperature, its haemolytic activity was completely absent at temperatures below 15°C.

By an immunofluorescence technique binding of DLF to erythrocytes was studied morphologically:

1. DLF was only bound to red cell ghosts (guinea pig and rat), but not to intact red cells. This binding was not temperature dependent.
2. Pretreatment of ghosts with SH-reagents such as NEM or PCMB did not prevent binding of DLF.
3. Ghosts prepared by different methods (hypotonic shock, freezing and thawing, ultrasonication, and resealing) were all able to bind DLF to their surface.

It is concluded that the binding of small amounts of DLF to intact red cells, observed by bioassay, was due to the presence of a small fraction of lysed cells, and that the binding to ghosts is not related to the lytic effect of DLF but secondary to lysis.

Key words: Direct Lytic Factor — Cobra Venom — Red Cells — Membranes — Haemolysis.

The direct lytic factor (DLF) of Naja naja venom is a basic polypeptide which acts on red cell membranes so as to render them susceptible to attack by phospholipase A. Thus, in combination with phospholipase A DLF is a potent haemolysin whereas it is weakly haemolytic only when acting alone (Condrea et al., 1964). Condrea et al. (1965) found that DLF is bound to red cells and more strongly to ghosts. The degree of binding to cells varied according to the species
and seemed to be correlated to the haemolytic sensitivity which differs considerably in different animal species. Vogt et al. (1970) suggested that DLF interacts with SH-groups of membrane proteins; a correlation between glutathione reductase activity of red cells and their susceptibility to DLF was found (Schroeter et al., 1972). We therefore attempted to study the nature of DLF binding in more detail. The results, described here, have led us to conclude that binding of DLF is not causative for but secondary to haemolysis.

**Material and Methods**

**DLF.** Highly purified DLF free of phospholipase A was prepared as described earlier (Lankisch et al., 1971, preparation DLF2).

**Antisera.** Rabbit anti-Naja naja venom serum: Solutions of 20 mg Naja naja venom (Ross Allen Reptile Institute) in 1 ml 0.1 M phosphate buffer pH 6.8 were mixed with 0.2 ml of 1% glutardialdehyde and left for 2 h at room temperature. In these cross-linked venom preparations the toxins are largely inactivated but well immunogenic (Brade and Vogt, 1971). After emulsifying with an equal volume of complete Freund's adjuvant, the preparations were injected i.m. into rabbits applying two doses of 10 mg each on two successive days. The rabbits were boosted 4 times with the same amount of cross-linked venom at intervals of two weeks. Blood was taken after ten weeks by heart puncture and the antiserum checked by agar immune electrophoresis. It developed a single, dense precipitation line against purified DLF.

Fluorescent goat anti-rabbit γ-globulin was obtained from Hyland, Costa Mesa, California.

**Red Cells and Ghosts.** Fresh, heparinized guinea-pig blood was centrifuged, the cells were washed three times in 0.15 M NaCl solution, and suspended in 0.01 M phosphate buffer containing 0.15 M NaCl (phosphate-buffered saline). Portions of these cell suspensions were osmotically lysed and served to prepare ghosts according to the procedure of Dodge et al. (1963). The cell concentrations used for binding studies were the same as in the original blood. For haemolytic assays suspensions of 10^9 cells/ml were used.

**Binding of DLF.** The binding of DLF to intact red cells or ghosts was evaluated by a functional assay and by immunochromical identification.

Estimation of the degree of DLF binding by bioassay: Suspensions of intact cells or membranes in phosphate-buffered saline were incubated with DLF at a final concentration of 3.3 \cdot 10^{-4} g/ml, at various temperatures. After various time intervals portions of the suspension were centrifuged and the residual DLF concentration was estimated by haemolytic assay on other guinea-pig red cells. For calibration various known dilutions of DLF were incubated simultaneously with portions of the same batch of red cells. The residual DLF activity found in the experimental supernatants was read from a calibration curve derived from the values of haemolysis obtained with the standard doses. The amount bound was then calculated. Haemolytic assays were carried out as described (Lankisch et al., 1971).

Detection of DLF by immune fluorescence microscopy: Suspensions of red cells or ghosts (1 ml) treated with DLF (3.3 \cdot 10^{-5} g/ml) at 37°C or in an ice bath for 30—90 min were centrifuged for 5 min and washed three times in ice-cold phosphate-buffered saline. They were then resuspended in the original volume of