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HAEMOLYTIC ASSAY FOR THE DETECTION OF ADENYLATE CYCLASE TOXIN OF *BORDETELLAPERTUSSIS*

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ABSTRACT

The aim of this study was to device a simple, sensitive and convenient assay for the detection of toxicity of the Adenylate cyclase toxin (ACT), a newly recognized toxin of *Bordetella pertussis.* Haemolytic activity of ACT was analysed as an assay and sheep RBCs were found to be suitable to detect the toxic effect down to the dose of 6.25 nmol cAMP/min/ml enzymic activity applied. The haemolytic activity was found to be dose-dependent.

Introduction

Toxic AC is capable of penetrating into target cells where it is activated by a host cell protein, calmodulin, and catalyzes the generation of uncontrolled CAMP by using host cell ATP (Confer and Eaton., 1982). Previous studies described the toxic effect of AC on various cell types, including human PMNs and macrophages (Confer and Eaton., 1982), lymphocytes, (Hanski and Farfel., 1985), monocytes, CHO cells, mouse S49 lymphoma cells, isolated rat pituitary cells (Hewlett et al., 1985; Gentile et al., 1988), and red blood cells (Rogel et al., 1991). The bases of evaluation of toxicity in these assays involved the demonstration of high levels of CAMP in the target cells, which, although reliable, is not simple and convenient. Therefore, a more direct approach was sought to detect the toxic activity of AC by using sheep and rabbit red blood cells to analyze haemolytic activity of AC on these cells.

Materials and Methods

Culture of organisms and purification of Adenylate cyclase

B. pemtssis strain BP348 (pRMB1) was grown in modified SS medium (Imaizumi et al., 1983). AC toxin was purified by urea extraction, calmodulin agarose affinity chromatography as described by Bellalou et al. (1990). Adenylate cyclase enzymic activity was measured according to the method of Solaman et al. (1974). The purified AC

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was dialysed extensively at 4 degree centrigrade (C) against 100 mM Tris-HCI pH 7.5,10 mM NaCl, and 1 mM $CaCl_2$.

Haemolytic assay

The blood was obtained from Beckton-Dickinson and centrifuged at 5000 rpm at room temperature for 3 min. The pallet was washed three times in haemolysis buffer (buffer H; 10mM Tris-HCl pH 7.5, 100 mM NaCl and 1mM ($CaCl_2$) and resuspended in buffer H to give final concentration of 2%. Neat and 2-fold dilutions of purified AC (representing 100 nmol cAMP/min/ml AC enzymic activity in a total volume of 50 microhtre of H buffer and its 2-fold dilutions) were used. Dilutions were prepared in duplicate in a round bottom microplate. Equal volumes of 2% sheep and rabbit RBCs were added to the wells. The plate was incubated at 37 degree C for 12-15 h in a humified box. The haemolytic activity after incubation was measured by transferring with great care, 30 micro litre of the supernates into another empty microplate. This plate was read on an ELISA reader (Anthos 2001) at A540. The assay was performed in duplicate and the mean was calculated.

Result

Haemolytic activities of ACT on Sheep and Rabbit RBCs

Haemolytic activity of calmodulin - affinity purified ACT was determined. In this assay, sensitivity of two species of RBCs towards ACT was investigated. Purified ACT (100 nmol cAMP/min/ml AC enzyme activity) and 2.fold dilutions of this activity were incubated with 2% SRBCs as detailed under Materials and Methods.

The results (see Fig.) showed a clear linear relationship between % haemolysis and the toxin dilutions. The rabbit RBCs were found fragile and some autolysis was observed in control cells. Sheep RBCs, on the other hand were found to be sensitive enough to detect haemolytic activity of ACT down to the level of 6.25 nmol/min/ml AC enzymic activity applied. The AC toxic activity on RBCs was found dose - dependent.

Discussion

The data presented in this study concerned an investigation of the haemolytic activity as an assay for toxicity of ACT. Haemolytic activity of ACT on sheep and rabbit RBCs was explored and sheep RBCs were found to be more sensitive. 80% haemolytic activity was observed with a dose of 60 nmol cAMP/min/ml AC enzymic activity. Taken together, these findings suggest that haemolytic activity. Taken together, these findings suggest that haemolytic activity of ACT could be used as an assay for determination of toxic activities in *B. penussis* ACT.

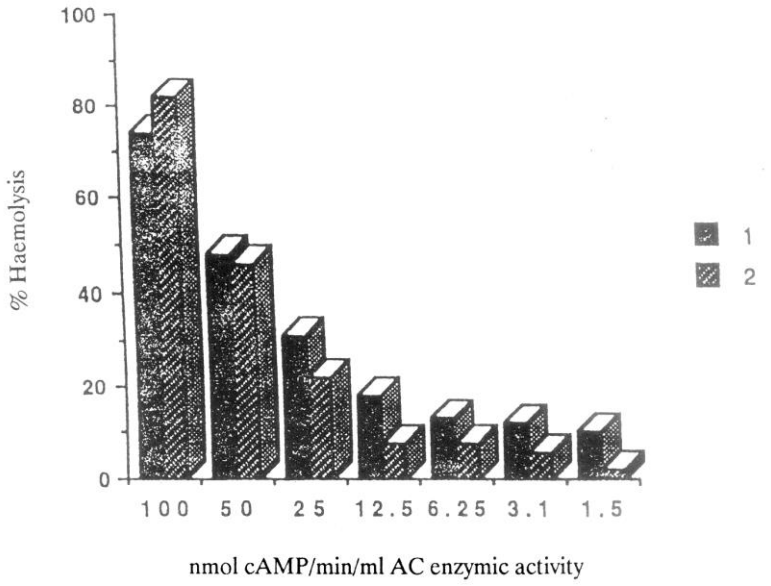

Fig. Determination of haemolytic activity of ACT on Sheep RBCs and its comparison with rabbit RBCs for their sensitivity towards ACT.

Several studies have reported that ACT increases the cAMP concentrations in a wide range of eukaryotic cells. It is because ACT has little target cell specificity. ACT also inhibits the cytotoxicity of non-phagocytic immune effector cells, e.g., natural killer (NK) cells (Hewlett et al., 1983). The basis of determination of toxicity in these cells is the measurement of the accumulated intracelluar cAMP. This involves the use of radioactive isotopes in the form of radiolabelled cAMP in a competitive binding assay (Gilman, 1970). Although this assay provides a standard and sensitive determination of ACT toxic activity, the methodology involved is laborious and time- consuming. Moreover, involvement of radioactive isotopes make its use expensive and potentially hazardous and confined to authorized persons.

ACT has been reported as a bi-functional protein exhibiting both CaM-sensitive ACT enzymic and haemolytic activities (Gaser et al., 1988). A single Tn5 insertion mutation in the rya operon resulted in a mutant BP348, deficient in both AC enzymic and haemolytic activity (Weiss et al., 1983). Both activities were restored when BP348 was complemented with plasmids carrying the complete cya operon (Brownlie et al., 1988). These data indicate that the haemolytic activity has a direct impact on toxicity and that

this activity could be used to determine the toxicity of ACT. RBCs are the most convenient cells for assaying cytolytic toxins because they are readily available, and contain a built-inmarker in the form of haemoglobin (Bernheimer, 1988). Species of RBCs used in an assay might be of importance since human RBCs have been shown to be an excellent target for internalization of AC (Masure et al., 1991) but not for haemolysis (Rogel et al, 1991). For this reason, two different species of RBCs were analysed in the present assay and found that sheep RBCs are sensitive targets and thus the haemolytic assay of ACT on sheep RBCs could be a useful and safe alternate for the detection and determination of ACT.

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