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Haemolysis of rabbit erythrocytes by a lectin from the seeds of *Croton tiglium*

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Abstract. The kinetics of haemolysis of rabbit erythrocytes by *Croton tiglium* lectin was studied as a function of concentration of the lectin and erythrocytes. The length of the prelytic period decreased with increasing lectin concentrations, indicating that the secondary events at the membrane which follow the binding of the lectin to cell surface carbohydrate receptors are accelerated at higher surface concentrations of the lectin. The rate or extent of haemolysis was not affected by the inclusion of ions like K^+, Ca^{2+} and Mg^{2+} in the medium or by the substitution of ionic medium by a non-ionic medium. The inhibition of haemagglutination and haemolysis of rabbit red cells by *Croton tiglium* lectin by antilectin rabbit serum was observed. A possible mechanism of haemolysis by the lectin is discussed.

Keywords. Lectin; haemolysis; *Croton tiglium*

Introduction

Proteins with haemolytic activity, or in general with cytolytic activity, have been of much interest to membrane biologists, since the study of the mode of action of such cytolytic agents has contributed considerably to the understanding of complexities of biomembrane organization and function (Alouf, 1977). Erythrocytes, the most well understood of all animal cells, have served as a convenient tool for the assay of lytic potency of cytolytic agents as well as for the elucidation of lytic mechanism. The intracellular composition of animal red cells differs markedly from that of the surrounding milieu. Thus, they exist in a steady state far from thermodynamic equilibrium and their viability depends on the ability of the erythrocyte membrane to selectively include certain components and exclude others (Sachs *et al.,* 1975). Haemolytic proteins bind or enzymatically degrade specific membrane-components like lipid or protein, thereby affecting membrane organization in such a way that it fails to serve as a selective permeability barrier. There is intrusion of water resulting in swelling and the erythrocyte is transformed from its normal discocyte form to a taut sphere. The cell ultimately bursts with the leakage of the cytoplasmic material into the medium. Although there is wide diversity in the pathways of haemolysis induced by various cytolytic proteins, the colloid osmotic swelling is in most cases the last step. Bacterial haemolysins like staphylococcal α-toxin, staphylococcal β-toxin and thiol-active haemolysins like streptolysin *O,* which are among the more well-known haemolysins, illustrate the diversity in haemolytic mechanisms. Staphylococcal α-toxin, a surface-active protein induces haemolysis by interfering with the hydrophobic region of the membrane,

staphylococcal β-toxin induces lysis by virtue of its phospholipase *C* activity specific for sphingomyelin, and streptolysin *O* by disorganizing membrane structure by binding specifically to cholesterol. However, there is usually a series of membrane events, more or less obscure, which precede haemolysis. The study of these events, which intervene the primary process of interaction of cytolytic agents with specific membrane components and the final loss of permeability properties, is expected to throw light on the question of how protein and lipid are woven into a structure which imparts the membrane physical stability and biological function.

All hitherto known cytolytic proteins have been isolated from either animal or bacterial sources. Although the presence of haemagglutinating and haemolytic proteins in the seed extract of *Croton tiglium* (Fam. *Euphorbiaceae)* has been known for a long time (Osborne, 1924; Karrer *et al.,* 1925; Stirpe *et al.,* 1976; Lin and Huang, 1976), the activities have been purified only recently. Banerjee and Sen (1981) reported the purification of a lectin from *C. tiglium* seeds with haemagglutinating activity towards erythrocytes of sheep, cow and a few other animals and haemagglutinating as well as haemolytic activity towards rabbit erythrocytes. The lectin purified by ion-exchange chromatogrphy and gel filtration, was found homogeneous by polyacrylamide gel electrophoresis at pH 4.5 and at pH 8.3 and also by immunodiffusion and Immunoelectrophoresis. That the haemagglutinating and haemolytic activities were associated with the same protein was demonstrated by the co-purification of the two activities, the simultaneous loss of the two activities on heating and on exposure to acid and alkaline pH values, and also by the ability of sheep erythrocytes to adsorb the haemolytic activity towards rabbit red cells. Although the lectin was not inhibited by simple sugars, both haemagglutination and haemolysis were inhibited by trypsin released glycopeptides from sheep red cell surface and pronase-digested trypsin fragments, suggesting that binding of the lectin molecule to carbohydrate receptors on rabbit erythrocytes surface was involved in inducing both haemagglutination and haemolysis. The carbohydrate nature of the receptor of *C. tiglium* lectin was further confirmed by the marked decrease in sensitivity of rabbit red cells pre-treated with D-galactose-binding lectins like *Abrus precatorius* agglutinin and *Ricinus communis* agglutinin towards *C. tiglium* lectin induced haemolysis. These results demonstrated that the primary processes in haemagglutination and haemolysis were associated with the specific binding of *C. tiglium* lectin molecules with rabbit erythrocyte carbohydrate receptors. However, the differential sensitivity of trypsinized rabbit erythrocytes to *C. tiglium* lectin-induced haemagglutination and haemolysis as well as different effects of temperature on the two phenomena, suggested that the secondary membrane events leading to agglutination and lysis followed different pathways. It will be interesting to know how the binding of *C. tiglium* lectin molecules to cell surface glycoprotein or glycolipid receptors triggers of events which lead to lysis.

In the present communication, we present the kinetics of haemolysis as a function of lectin concentration and red cell concentration and also the effect of changing milieu composition on haemolysis. The inhibition of haemolytic and haemagglutinating activities of *C. tiglium* lectin by rabbit antiserum is also discussed in this paper. These information may lead to a better understanding of secondary membrane events.

Materials and methods

Preparation of protein solutions

The lectin was isolated and purified from *C. tiglium* seeds as described by Banerjee and Sen (1981). The concentration of the purified lectin was determined spectrophotometrically at 280 nm using a value of $E^{1\%}_{1cm} = 15.6$ dl g^{-1}.

Purification of IgG from rabbit antiserum and preparation of Fab fragment

The isolation of IgG from rabbit antiserum against purified *C. tiglium* lectin was carried out according to Fahey and Terry (1967). Twenty five ml of rabbit antiserum dialysed against 0.015 M potassium phosphate buffer, pH 8.0 was applied on a column (28 × 2.8 cm) of DEAE-cellulose equilibrated with the same buffer. Fractions which eluted out during the washing of the column with the starting buffer contained purified IgG with antilectin antibody activity and were pooled and precipitated by $(NH_4)_2\ SO_4$ at 80% saturation.

The degradation of IgG to Fab fragment was carried out according to Porter (1959). Ten ml of IgG solution containing 150 mg protein in 0.1 M sodium phosphate buffer, pH 7.0 containing 0.01 M cysteine and 2 mM EDTA were incubated with 1.5 mg papain. After incubation, the reaction mixture was exhaustively dialysed against water during which the F_c fragment crystallized out. The clear solution obtained after centrifugation of the dialysate, was subjected to chromatography on Sephadex G-200 when the Fab fragment emerged as a large symmetrical peak.

Assay of haemolytic activity

This was carried out according to Kabat and Mayer (1967). Rabbit erythrocytes washed 3–4 times with 0.01 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.0 were made into an approximately 6% suspension (v/v) or in any other isotonic buffered medium such as 0.3 M D-glucose or 0.3 M sorbitol buffered with 0.01 M sodium phosphate, pH 7.0. The suspension was filtered through a thin bed of adsorbent cotton to remove any clumps and diluted with phosphate buffered saline so that the final suspension on complete lysis with 20 vol. of distilled water gave an absorbance of 0.60 at 541 nm. This corresponds to a 6% suspension (v/v). Five millimeters of this suspension was incubated at 25° C with an equal volume of lectin solution containing 0.2–10 µg of lectin. Aliquots of 0.2 ml of reaction mixture were withdrawn at definite time intervals, diluted to 2 ml with chilled PBS and centrifuged immediately in the cold. The absorbance of the supernatant was read at 541 nm against a blank without lectin. Haemolytic activity was expressed as percentage of red cells lysed under the conditions of assay.

Results

Kinetics of haemolysis of rabbit erythrocytes as a function of lectin concentration

Figure 1 shows the time-course of haemolysis of a 3% suspension of rabbit erythrocytes as a function of different lectin concentrations in the range of 0.1–5 µg/ml. It will be noted from the figure 1 that the 'haemolysis *vs*. time' course is sigmoidal and is characterized by three distinct regions. These are, (i) a prelytic period or

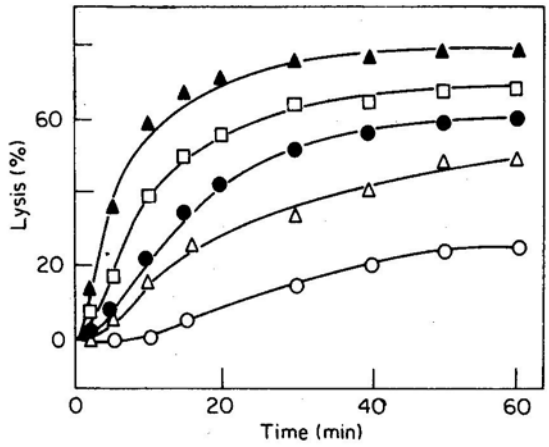

Figure 1. Kinetics of haemolysis of a 3% suspension of rabbit erythrocytes in 0.01 M sodium phosphate buffer containing 0.15 M NaCl (PBS) at different concentrations of *C. tiglium* lectin at 25°C. (O), Per cent haemolysis at 0.1 µg/ml; (Δ), 0.5 µg/ml;(●), 1 µg/ml; (c), 2 µg/ml and (▲), 5 µg/ml lectin concentration.

lag phase during which there is relatively little or no haemolysis, (ii) a period of rapid haemolysis during which there is almost a linear relation between per cent haemolysis and time and (iii) a static phase during which there is no further lysis. The sigmoidal nature of the curve indicates that the binding of the lectin molecule to rabbit erythrocyte surface and the final act of haemolysis are intervened by a series of membrane processes. The kinetics of the secondary membrane processes appear to be reflected in the length of the prelytic period. It will be seen from figure 1 that the length of the lag phase which is hardly detectable at lectin concentrations above 1 µg/ml, decreases with increasing lectin concentration. This suggests that the secondary membrane processes are accelerated at higher concentrations of the lectin molecules on the rabbit erythrocyte surface.

Kinetics of haemolysis of rabbit erythrocytes as a function of erythrocyte concentration

Figure 2 shows the time-course of haemolysis of rabbit erythrocytes at a lectin concentration of 1 µg/ml at different erythrocyte concentrations in the range 2–10%. Figure 2 (A) shows the plot of absorbance at 541 nm, which is a direct measure of the number of cells lysed against time, while figure 2 (B) shows the rate of per cent haemolysis, a normalized quantity which takes into account the total number of cells present. It will be seen from figure 2 (A) that almost the same number of cells are lysed during the first five min, irrespective of the total number of cells present. A low cell surface density of lectin molecules resulting from the presence of a large number of red cells competing for a given amount of the lectin delays the onset of lysis, and this explains the observed insensitivity of the initial rate of per cent haemolysis to the number of cells present. It will be further noted that the rate as well as the extent of per cent haemolysis is maximum at 2% red cell concentration and minimum at 10% concentration. This is due to the fact that there is a larger percentage of red cells with the optimum number of lectin molecules bound on them, when a relatively fewer of the latter are present.

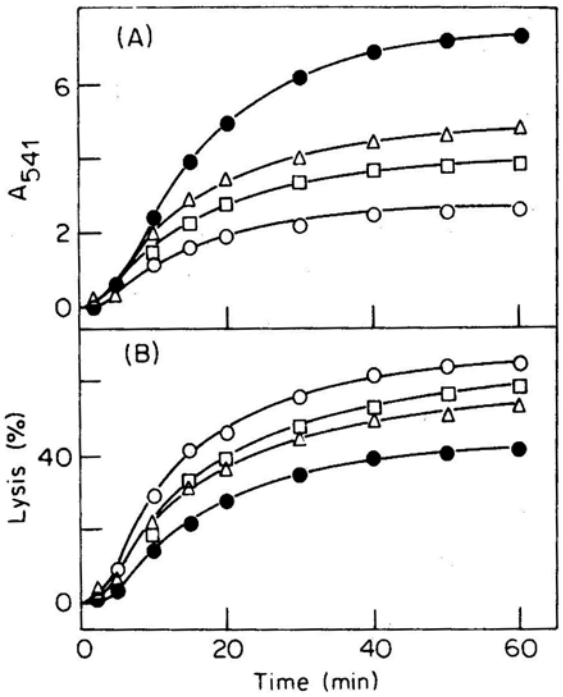

Figure 2. Kinetics of haemolysis of rabbit erythrocytes at a lectin concentration of 1 μg/ml at different erythrocyte concentrations, (A) shows the plot of absorbance at 541 nm, and (B) shows per cent haemolysis. (O), Absorbance and per cent haemolysis at 2% erythrocyte concentration; (c), 3% concentration; (Δ), 5% concentration; (●), 10% erythrocyte concentration.

Inhibition of haemagglutination of sheep erythrocytes and haemolysis of rabbit erythrocytes by rabbit antilectin antiserum and Fab from antilectin IgG

Table 1 shows the effect of preincubation of *C. tiglium* lectin solution with rabbit serum and antilectin rabbit serum on its haemagglutinating and haemolytic activeties. It will be noted that the haemolytic activity of *C. tiglium* lectin towards rabbit

Table 1. Inhibition of *C. tiglium* lectin-induced haemagglutination of sheep erythrocytes and haemolysis of rabbit erythrocytes by normal rabbit serum, antilectin antiserum and Fab fragment.

	Dilution or concentration completely inhibiting 4 minimum haemagglutinating dose	Dilution or concentration completely inhibiting haemolysis of rabbit erythrocytes by lectin at a concentration of 1 μg/ml[a]
Normal serum	No inhibition with undiluted serum	No inhibition with undiluted serum
Antilectin antiserum	1:8	1:10
Fab fragment from antilectin rabbit IgG	200 μg/ml	200 μg/ml

[a] Per cent haemolysis of a 3% suspension of rabbit erythrocytes was monitored after 10 min of incubation.

red cells monitored after 10 min was almost totally inhibited by rabbit antiserum at a dilution of 1 : 10, while the haemagglutinating activity towards sheep red cells is inhibited at a dilution of 1 : 8. No effect was observed with serum from non-immunized animals. To exclude the possibility that the inhibition of haemolysis and haemagglutination arose as a result of the removal of the lectin from the solution by the formation of insoluble antigen-antibody complex and was thus only apparent, experiments were done with the Fab fragment of the antilectin antibody. Table 1 shows that the monovalent antibody, Fab fragment, is also potent in inhibiting haemagglutinating and haemolytic activity.

Since it has been shown that the haemagglutinating and haemolytic activities of the lectin are primarily associated with the carbohydrate-binding function, it appears that at least some of the antibody-combining sites of the lectin are either the same with, or are in close proximity to the sugar-binding site of the lectin.

Effect of EDTA, ions K^+, Ca^{2+} and Mg^{2+} on the kinetics of haemolysis

Figure 3 shows that the substitution of 0.01 M sodium phosphate in PBS by 0.01 M Tris-HCl, pH 7.0, the incorporation of 10 mM Ca^{2+}, Mg^{2+}, or K^+ or 1 mM EDTA did not affect the kinetics of haemolysis. The results indicate that divalent cations like Ca^{2+}, Mg^{2+} are not implicated in either the binding of the lectin molecule to cell surface or in the secondary membrane events.

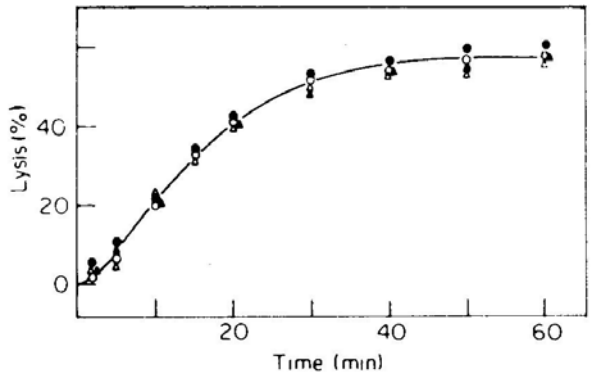

Figure 3. Effect of changing ionic composition on the rate and extent of haemolysis of rabbit erythrocytes at a lectin concentration of 1 µg/ml of 25° C. (O), Per cent haemolysis of rabbit erythrocytes in PBS; (●), 0.01 M Tris-HCI buffer, containing 0.15 M NaCl and 0.01 M $CaCl_2$, pH 7.0; (Δ), 0.01 M Tris-HCl buffer containing 0.15 M NaCl and 0.01 M $MgCL_2$, pH 7.0; (▲), 0.01 M Tris-HCl buffer containing 0.15 M NaCl and 0.001 M EDTA, pH 7.0.

Effect of non-ionic environment of the rate of haemolysis

Figure 4 shows the haemolysis of a 3% suspension of rabbit erythrocytes by *C. tiglium* lectin at a concentration of 1 µg/ml at 25° C in isotonic-D-glucose and sorbitol buffered with 0.01 M sodium phosphate buffer pH 7.0. It will be noted that haemolysis is almost completely inhibited in isotonic glucose solution. However, there was no change in the minimum haemagglutinating dose. Furthermore, if rabbit erythrocytes in isotonic glucose were incubated for 10 min with *C. tiglium* lectin at a concentration of 1 µg/ml and subsequently washed and resuspended in PBS, haemolysis took place. This showed that while haemolysis was

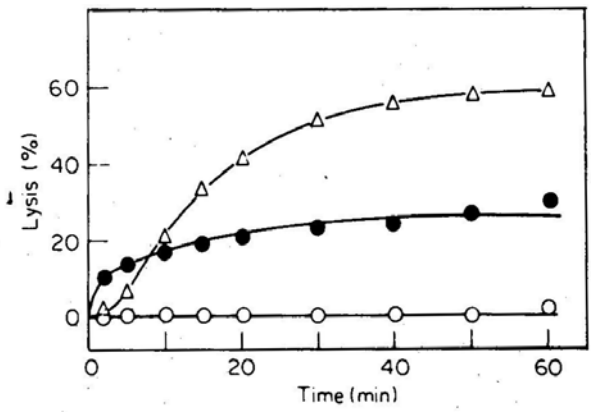

Figure 4. Per cent haemolysis of rabbit erythrocytes at 1 µg/ml lectin concentration in non-ionic environment. (O), Per cent haemolysis of rabbit erythrocytes incubated in 0.30 M D-glucose buffered with 0.01 M sodium phosphate, pH 7.0; (Δ), 0.30 M sorbitol solution buffered with 0.01 M sodium phosphate, pH 7.0; (●), per cent lysis of rabbit erythrocytes which were incubated for 10 min at 1 µg/ml lectin concentration in isotonic D-glucose solution washed twice with isotonic D-glucose solution and finally resuspended in PBS.

inhibited in the pesence of 0.1 M glugose, the binding of the lectin molecule to rabbit red cells and their agglutination were not. These results indicate that an ionic environment is not essential for binding and haemagglutination.

The binding of the lectin molecule to rabbit red cell surface might induce changes in membrane permeability and like ionophores render them permeable to cations resulting in an imbalance of osmotic pressure across the erythrocyte membrane. Such a mechanism would preclude haemolysis in a non-ionic milieu. Alternatively, D-glucose which is readily transported across erythrocyte membrane (Nafatlin and Holman, 1977), might inhibit haemolysis by causing increase of viscosity of haemoglobin and thereby preventing its release through membrane lesions (Seeman, 1974). To eliminate one of these two possibilities rabbit erythrocytes were incubated with lectin in isotonic sorbitol, which has no such effect on haemoglobin. Figure 3 shows that there is no significant change either in the extent or kinetics of haemolysis in isotonic sorbitol, implying that ionic environment is not essential for haemolysis.

Further, the absence of lag phase and completion of the process of lysis within 10 min, when cells previously incubated with lectin in D-glucose are exposed to PBS indicate that secondary membrane processes essential to haemolysis proceed uninterrupted in 0.3 M D-glucose. Thus, it is apparent that the observed inhibition of haemolysis in D-glucose is superficial and has no bearing either on the primary event of lectin cell surface interaction or on the secondary membrane processes.

Discussion

In this paper we have presented the results of our studies on the effect of various parameters on the haemolysis of rabbit red cells by *C. tiglium* lectin in continuation of our previous report (Banerjee and Sen, 1981) which described the purification and physicochemical properties of the lectin and also established the involvement

of the carbohydrate binding site in inducing both haemagglutination and haemolysis.

Since it was established that *C. tiglium* lectin induces haemolysis by binding to rabbit red cell surface carbohydrate receptors, it is interesting to consider how the interaction of the lectin with cell surface glycoprotein or glycolipid can trigger off events which lead ultimately to cell lysis. Although lectins are known to affect cell surface morphology and to induce complex biochemical events like agglutination and mitosis by binding specifically to carbohydrate receptors (Goldstein and Hayes, 1978), no lectin has been shown to have cytolytic activity. In other words, it is unlikely that the loss of structural integrity of the rabbit red cell membrane results directly from alterations in topography or mobility of membrane proteins induced by binding of the lectin to carbohydrate receptors. The involvement of the secondary membrane events is suggested by the study of the kinetics of haemolysis (figure 1) which indicates that there is a finite time interval (lag phase) between the binding of the lectin molecules and the escape of haemoglobin. Further the length of this lag phase which is a measure of the kinetics of the secondary membrane events yet to be defined, is a function of the concentration of lectin-receptor complex (figures 1 and 2). These secondary events are, however, not identical with those leading to haemagglutination as shown by the differential response of trypsinized rabbit red cells to *C. tiglium* lectin-induced haemagglutination and haemolysis as well as by the different temperature-sensitivity of the two processes (Banerjee and Sen, 1981). Thus, while agglutination follows from cross-linking of adjacent cells through polyvalent lectin molecules, which may be preceded by redistribution of lectin-receptor complexes to form clusters or patches (Inbar and Sachs, 1973), haemolysis follows from altogether different membrane events. In view of these considerations and the requirement of phospholipase or protease activity to induce haemolysis (Alouf, 1977), it is not unlikely that binding of *C. tiglium,* lectin to cell surface glycoprotein might stimulate phospholipase or protease activity. There are reports that certain lectins stimulate enzymatic activity of membrane proteins. The effect may be an indirect one, resulting from lectin-induced membrane perturbations, *e.g.* the stimulation of galactosyl-transferase activity by binding of concanavalin A to Golgi membranes (Young *et al*.,1976) or may result directly from lectin-glycoprotein interaction as in the case of the stimulation of β-D-galactoside α2→ 6-sialyltransferase of bovine colostrum by bovine heart lectin (Scudder *et al.*, 1982).

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