Note

Induction of Superoxide Production in Polymorphonuclear Leucocytes by *Ricinus* Lectins and Its Dependency on Lectin Valency

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Received September 24, 1985

Polymorphonuclear (PMN) leukocytes undergo a cyanide-insensitive increase in oxygen consumption upon perturbation of the receptors on the cell surface, concomitant with various metabolic alterations such as the production of superoxide and stimulation of hexose monophosphate shunt activity.^{1~3} Plant lectin is one of the reagents which induce alterations in a number of diverse biological activities of the cells by binding to the carbohydrate moiety on the cell surface. In fact, concanavalin A binds to the mannose- or glucose-containing oligosaccharide moieties on the surface of PMN leukocytes and induces superoxide production.^{4,5}

Ricinus lectins, hemagglutinin (CBH) and ricin D, are composed of an A-chain, which inactivates eukaryotic ribosomes, and a B-chain, which binds to a galactose-containing saccharide. The B-chain of CBH has one high-affinity saccharide (HA-) binding site, whose K_a for lactose at 25°C is $1.93 \times 10^4 \text{ M}^{-1}$,⁶ while the B-chain of ricin D has one HA-binding site, whose K_a is $1.86 \times 10^4 \text{ M}^{-1}$, and a low-affinity saccharide (LA-) binding site, whose K_a is $0.3 \times 10^{-4} \text{ M}^{-1}$.⁷ Since CBH and ricin D have the subunit structure of a heterotetramer (AB)₂ and a heterodimer (AB), respectively, CBH binds divalently to the receptors on the cell surface with two high-affinity binding sites, while ricin D binds divalently with high- and low-affinity binding sites. The isolated B-chain retained full saccharide binding-ability and was easily cross-linked to form a dimer through the cysteine residue, which participates in the interchain disulfide bridge. Thus *Ricinus* lectins are well suited for an investigation of the effects of the number of saccharide binding sites (valency) and the saccharide-binding affinity of the lectin on the induction of superoxide production in PMN leukocytes.

In this paper, we describe the superoxide production in

PMN leukocytes by *Ricinus* lectins, and its dependency on the valency and saccharide-binding affinity of the lectins.

Ricinus lectins and their B-chains were prepared as described earlier.^{8,9} The B-chain dimer of CBH was prepared by cross-linking the reduced B-chains using *N,N'*-*o*-phenylenedimaleimide as described previously,¹⁰ and that of ricin D by auto-oxidation of the reduced B-chain.⁸ These were purified by gel filtration on a Sephacryl S-200 column. PMN leukocytes were prepared from guinea pigs by the method of Sbarra and Karnovsky.¹¹ Twenty ml of 1% casein solution in 0.9% NaCl-10 mM Tris-HCl buffer, pH 7.4, was intraperitoneally injected into guinea pigs (500~600 g). After 15~16 hr, peritoneal cells were harvested using Krebs-Ringer-phosphate (KRP) solution, pH 7.4, containing 0.5 mM CaCl₂, centrifuged, and washed twice with the same buffer. PMN leukocytes were freed from contaminating erythrocytes by a brief hypotonic shock and then resuspended in KRP solution. The superoxide anions released from the PMN leukocytes were measured by the reduction of ferricytochrome C by the method of Cohen *et al.*¹² To 2 ml of the cells suspension (2×10^6 cells/ml) in KRP solution incubated previously with 10 μ l of 2 mM ferricytochrome C (Sigma Chemical Co.) solution at 37°C, 20 μ l of the lectin solution was added and incubated at 37°C. The reduction of cytochrome *c* was monitored by the increase in absorbance at 550~540 nm using a dual-wavelength autorecording spectrometer (Shimadzu Co., Ltd., type UV-300) with a thermostatically controlled cuvette holder and an acrobat stirrer. An oxygen electrode was attached to the thermostatically controlled glass vessel at 37°C. Agglutination of PMN leukocytes by lectin was measured by the reduction in the absorbance at 610 nm after adding 20 μ l of the lectin solution to 2 ml of the cell suspension (3.3×10^6 cells/ml). Cytoagglutinating potency was compared by measuring the reduction in the absorbance at 610 nm after 5 min-incubation.

When PMN leukocytes were incubated with CBH at 37°C, the oxygen uptake by the cells began to increase immediately after the addition of CBH and increased almost linearly with the elapse of incubation time (Fig. 1A), and almost simultaneously the superoxide production was induced (Fig. 1B). Both of these PMN responses were dependent on the CBH concentration, and reached a plateau at about 50 μ g/ml. No additional change was observed at concentrations as high as 300 μ g/ml. To understand the effects of lectin valency on superoxide production, the amount of superoxide produced in a 5-min incubation with various concentrations of A-chain (no valency), B-chain (mono-valency), and B-chain dimer (divalency) of CBH, were measured. As shown in Fig. 2A, the A-chain and B-chain did not induce superoxide production, but the B-chain dimer produced superoxide to the

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FIG. 1. Changes in Oxygen Uptake (A) and Superoxide Production (B) Caused in PMN Leukocytes by CBH.

All cells were incubated in KRP solution at 37°C. Oxygen uptake by the cells (3.6×10^7 cells) was monitored by an oxygen electrode, and superoxide released from the cells (4×10^6 cells) was measured by reduction of ferricytochrome C ($A_{550-540 \text{ nm}}$) using a dual-wavelength auto-recording spectrometer.

FIG. 2. Superoxide Production in PMN Leukocytes Caused by *Ricinus* Lectins and Their B-Chain Monomer and Dimer. (A) is CBH and (B) is Ricin D.

Experimental conditions were as in Fig. 1. All protein concentrations were expressed as molarity. ●, intact lectin; ○, B-chain monomer; ○, B-chain dimer; ▲, A-chain.

same extent as CBH, indicating clearly that divalent binding of the lectin to PMN leukocytes is responsible for the induction of superoxide production.

Ricin D also induced superoxide production in a similar way to CBH, but the required amount of ricin D, which

FIG. 3. Changes in Agglutination of PMN Leukocytes Caused by CBH (A) and Comparison of Cytoagglutinating Potency of CBH and Ricin D (B).

Twenty µl of the lectin solution was added to 2 ml of PMN leukocyte suspension (3.3×10^6 cells/ml) and incubated at 37°C. Agglutination of the cells was monitored by the absorbance at 610 nm using an auto-recording spectrometer. Cytoagglutinating potency was compared by measuring the reduction in the absorbance at 610 nm after a 5-min incubation. ○, CBH; ●, ricin D.

binds to the cells divalently with high and low affinity, was much larger than that of CBH, which binds to the cells divalently with high affinity. This shows that not only divalent binding of the lectin to the cells but also its saccharide-binding affinity is important for superoxide production. Since the saccharide-binding affinity of the HA-binding site of ricin D is almost equal to that of CBH,^{6,7} it was suggested that the low potency of ricin D in the superoxide production may be due to low saccharide-binding affinity of its LA-binding site. The isolated B-chain of ricin D produced superoxide in a similar way to ricin D, whereas the B-chain dimer, which has two HA-binding sites and two LA-binding sites, had activity as high as the B-chain dimer of CBH, which has two HA-binding sites (Fig. 2B).

Di- or multi-valent binding of lectin to the cells induces cytoagglutination. The rate-limiting step of this cytoagglutination is receptor diffusion. This in turn is thought to be limited by membrane lipid fluidity and by the association of cytoskeletal elements.^{13,14} When CBH was added to 2 ml of PMN leukocytes suspension at 37°C, as shown in Fig. 3A, the cells agglutinated rapidly in the initial few minutes while the isolated B-chain of CBH did not agglutinate the cells. Ricin D also agglutinated the cells in a similar way to CBH, but its cytoagglutinating potency was very low; the amount of ricin D required to cause the same agglutination was approximately 60 times that of CBH. Thus the effects of valency and saccharide-

binding affinity of *Ricinus* lectins on the agglutination of PMN leukocytes strikingly resembled those of the superoxide production in these cells.

From these results, it was inferred that superoxide production in PMN leukocytes by *Ricinus* lectins could be induced by cross-linking the oligosaccharide containing receptors on the cell surface into clusters by their di-valent binding and resulted in the alteration of the membrane or cytoplasmic structure.

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