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RECOGNITION OF 2-KETO-3-DEOXYOCTONATE IN BACTERIAL CELLS AND LIPOPOLYSACCHARIDES BY THE SIALIC ACID BINDING LECTIN FROM THE HORSESHOE CRAB CARCINOSCORPIUS ROTUNDA CAUDA

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The sialic acid binding lectin carcinoscorpin agglutinates Escherichia coli K12 and Salmonella minnesota R595 cells. This interaction can be inhibited by the saccharides namely 2-keto-3-deoxyoctonate and the disaccharide O-(N-acetylneuraminyl)(2→6)2-acetamido-2-deoxy-D-galactitol. N-acetylneuraminic acid is shown to be a poor inhibitor. The same behaviour is seen when purified lipopolysaccharides from these two Gram negative bacteria are used. Vibrio cholerae, a Gram negative bacterium devoid of 2-keto-3-deoxyoctonate and Staphylococcus aureus a typical Gram positive bacterium failed to agglutinate in the presence of the lectin. The results suggest that the 2-keto-3-deoxy-octonate residues might represent the physiological substrate for the sialic acid binding lectin from the horseshoe crab.

INTRODUCTION

Previous work in our laboratory indicated the presence of a sialic acid binding lectin in the horseshoe crab Carcinoscorpius rotunda cauda available in the coastal areas of the Bay of Bengal. This lectin called carcinoscorpin has been purified to homogeneity (1), characterized with respect to its amino acid composition and subunit structure (2) and immobilized onto Sepharose(3,4). It has been shown that this lectin is antigenically unrelated to limulin, the sialic acid binding lectin from the horseshoe crab Limulus polyphemus (2). The biological significance of its presence in the hemolymph of the horseshoe crab has always remained an intriguing problem. This arthropod arose very early

ABBREVIATIONS USED : CSN-Carcinoscorpin ; KDO-2-keto-3-deoxyoctonate ; NANA-N-acetylneuraminic acid ; Disaccharide-O-(N-acetylneuraminyl)(2→6) 2-acetamido-2-deoxy-D-galactitol ; LPS-Lipopolysaccharide

KEY WORDS : SIALIC ACID BINDING LECTIN/2-KETO-3-DEOXYOCTONATE/ LIPOPOLYSACCHARIDES/RECOGNITION

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in evolution and fossilized remains can still be found dating back to several hundred millions of years. Absence of immunoglobulins and immunoglobulin - like molecules in the hemolymph have been well documented (5). In such a situation, the wandering phagocytic cells and some humoral recognition molecules in the hemolymph of these organisms have been suggested to be involved in the host defense mechanisms (6). In this communication we report that the interaction between the sialic acid binding lectin carcinoscorpin and bacterial lipopolysaccharides occure through the specific recognition of 2-keto-3-deoxyoctonate residues which are quite similar to sialic acid (7).

MATERIALS AND METHODS : Carcinoscorpin was purified according to the procedure described earlier (1,2). Protein was estimated by the method of Lowry et al (8) using crystalline bovine serum albumin as standard. NANA, KDO were procured from Sigma Chemical Co., USA. Preparation of O-(N-acetylneuraminyl)(2→6) 2-acetamido-2-deoxy-D-galactitol was according to a procedure described earlier (2). Carrier free ^{125}I was obtained from Bhaba Atomic Research Centre, Bombay.

Bacterial Strains : Salmonella minnesota R595 (heptose-less) was a kind gift from Dr. Manoranjan Singh, University of Texas Health Science Centre, USA. Escherichia coli K12 Row, Vibrio cholerae H218 (Biotype-classical), and Staphylococcus aureus PS 81 were available at the Department of Microbiology, IICB. Bacteria were grown in Brain Heart Infusion agar, 3% BHI, (Difcolabs, USA)and 15% agar (Oxoid, USA) in Roux flasks for 18 hrs at 37°C. The cells were harvested, washed twice and suspended in sterile saline (0.9%).

Isolation of lipopolysaccharide : Lipopolysaccharides from S. minnesota and E.coli cells were isolated according to the method of Galanos et al (9). Their KDO content was determined after hydrolysis in 0.05M H_2SO_4 at 80°C for 1 hr, according to the thiobarbituric acid method (10,11). The isolated lipopolysaccharides were found to be free of nucleic acids, as measured by their absorbance at 260nm.

Agglutination of bacteria : The method used is essentially as described by Lotan et al (12). Agglutination was followed at 580nm, in a final volume of 1 ml at room temperature (28-30°C). The incubation mixture contained 50mM Tris-HCl buffer pH 8.0, 100mM NaCl, 10mM $CaCl_2$ in the case of E.coli K12 and 5mM $CaCl_2$ in the case of S.minnesota R595 cells, varying amounts of carcinoscorpin and the bacterial cells enough to give an initial absorbance of around 0.5 at 580nm. Suspension of the cells in buffer containing calcium ions but without carcinoscorpin served as a control. Inhibition of bacterial agglutination was followed using the above conditions, in the presence of appropriate concentrations of KDO, NANA and the disaccharide.

Iodination of carcinoscorpin : This was carried out according to the earlier method (1). The iodinated lectin had a specific activity of 2800 cpm/ng. The radioactivity was measured in a Packard Autogamma 5110 counter.

Interaction of purified LPS with ^{125}I-Carcinoscorpin: The incubation mixture contained in a final volume of 100 µl, 50mM Tris HCl buffer pH 8.0, 100mM NaCl, 5mM $CaCl_2$, 23 µgs of CSN (0.05 nmoles), 2.2 x 10^4cpm of ^{125}I-CSN and varying amounts of S.minnesota R595 lipopolysaccharide, as expressed by its KDO content. The lipopolysaccharide suspension was sonicated for 1 min, in ice just before use. The tubes were kept at room temperature for 2 hrs and centrifuged at 3000 g for 10 min. The precipitate was washed twice with 200 µl

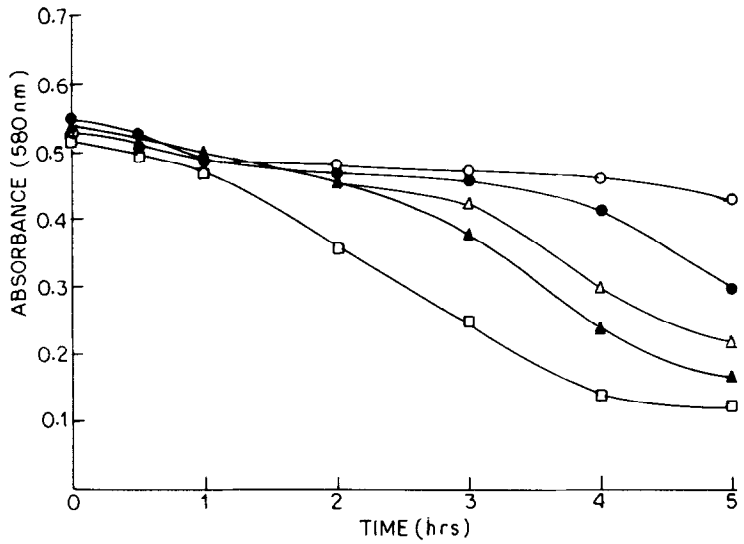

Figure 1. Agglutination of E.coli K12 cells by carcinoscorpin, at room temperature. The conditions were as described in the text. Agglutination was followed for 5 hrs in the presence of the lectin at (a) 10 μg/ml (●—●) ; (b) 50 μg/ml (△—△) ; (c) 100 μg/ml (▲—▲) ; (d) 150 μg/ml (□—□). Bacteria in buffer alone (○—○) served as a control.

of the above buffer containing calcium ions and the radioactivity was measured. Inhibitors of the interaction were included in the standard binding system, using a fixed saturating amount of S.minnesota R595 lipopolysaccharide (6 nmoles of KDO).

RESULTS AND DISCUSSION

Figure 1 shows the marked agglutination of E.coli K12 cells in the presence of increasing concentrations of carcinoscorpin, within a period of 5 hrs. Figure 2 represents the effect of saccharides, namely 4mM KDO and 0.14mM disaccharide, on the aggregation of E.coli K12 cells. Significant inhibition of agglutination could be seen in the presence of these inhibitors. Extensive agglutination of cells could also be seen when S.minnesota R595 was used, as shown in Figure 3. This figure also shows the marked inhibition of agglutination by 2mM KDO and 0.14mM disaccharide. When the experiment was performed in the absence of calcium ions, very feeble agglutination occurred. Also, it is interesting to note that NANA, which has a poor affinity for the sialic acid binding lectin (2,3) does not bring about a significant inhibition of aggregation of cells when compared to KDO and the disaccharide. In another experiment, it was found that no agglutination occurred over a period of 5 hrs when Vibrio cholerae was used, which as a species do not possess KDO in their lipopolysaccharide structure (13). The Gram positive Staphylococcus aureus cells also failed to agglutinate under identical conditions, when 120 μg/ml of CSN was used (figures not shown). A calcium concentration of 5mM was chosen for the

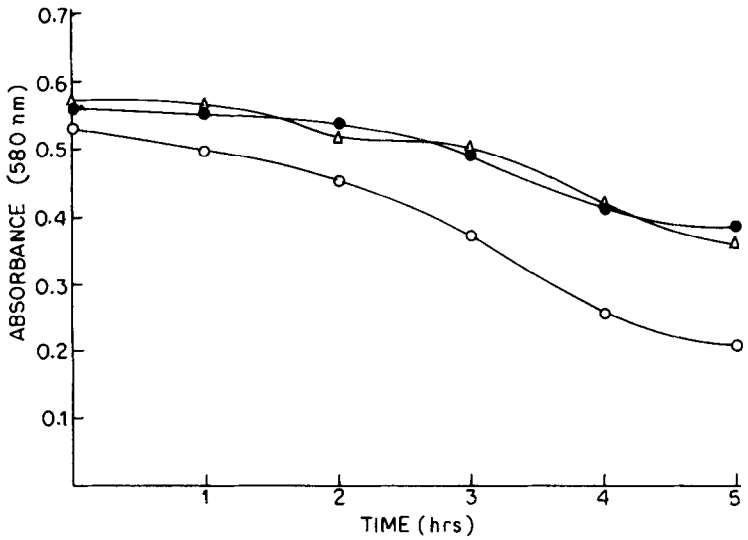

Figure 2. Agglutination of E.coli K12 cells in the presence of saccharide inhibitors. Final concentration of carcinoscorpin in the cuvettes was 100 µg/ml. (a) agglutination by lectin alone (o—o) ; (b) lectin + 4mM KDO (●—●) ; (c) lectin + 0.14mM disaccharide (Δ—Δ)

agglutination of S.minnesota cells instead of 10mM as in the case of E.coli K12 cells, due to a tendency of the former cells to aggregate at 10mM $CaCl_2$. Figure 4 represents the titration of 23 µgs of carcinoscorpin (0.05 nmoles)

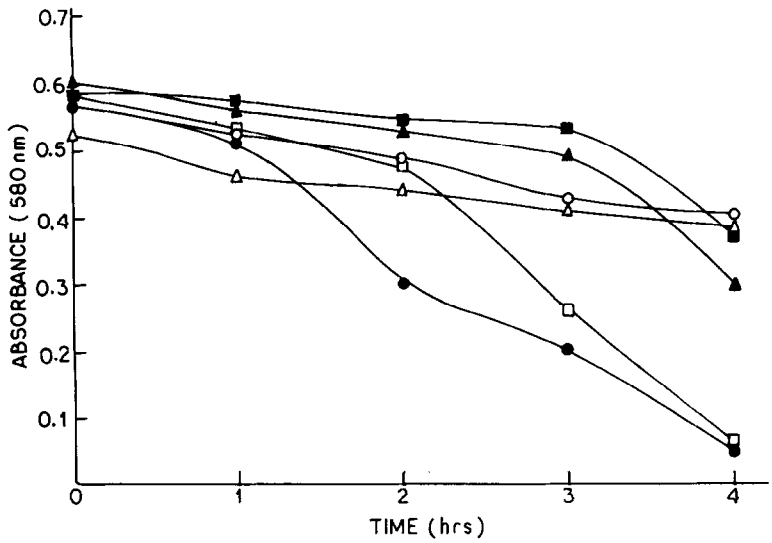

Figure 3. Agglutination of S.minnesota R595 cells by carcinoscorpin and the effect of inhibitors. Final concentration of the lectin in all the cuvettes was 120 µg/ml. (a) Bacterial cells in buffer alone (o—o) ; (b) cells in the presence of the lectin with calcium ions (●—●) ; (c) cells in the presence of the lectin without calcium ions (Δ—Δ) ; (d) cells + lectin + 2mM KDO (▲—▲) ; (e) cells + lectin + 2mM NANA (□—□) ; (f) cells + lectin + 0.14mM disaccharide (■—■).

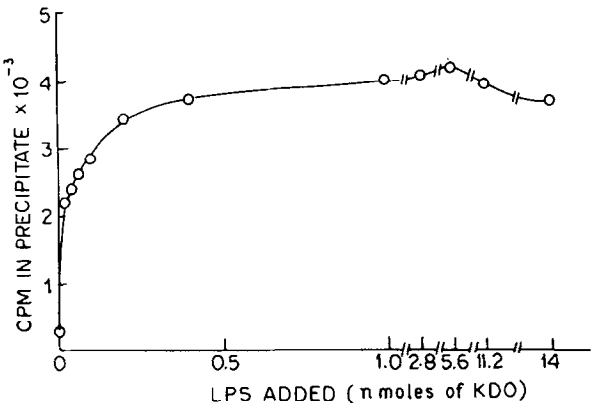

Figure 4. Interaction of the lipopolysaccharide from S.minnesota R595 with carcinoscorpin. The details of the experiment are described in the text.

with increasing concentrations of S.minnesota R595 LPS, expressed as its KDO content. The molar ratio of CSN to KDO in the titration was varied from 2:1 to 1:280. As can be seen from the figure, at the saturation point which corresponds to approximately 6 nmoles of bound KDO, only 20% of the added counts could be precipitated. The same behaviour could be seen with E.coli K12 lipopolysaccharide also. Under identical conditions, if 25 µgs of fetuin is added instead of LPS, 85% of the added radioactivity was recovered in the precipitate. Any attempt to increase the percentage of binding by solubilizing the isolated LPS in 0.1% Triton X-100 (14) or by triethylamine treatment (15) or by alkali treatment (16) were unsuccessful, as there is a tendency of the lipopolysaccharide to aggregate the moment calcium ions are introduced. We infer that this low binding of carcinoscorpin to S.minnesota R595 LPS or to E.coli K12 LPS is due to their self-aggregating tendency in the presence of calcium ions (17) and that this phenomenon dictates the availability of KDO residues for binding to the lectin. Hence a better picture of the KDO interaction with the lectin is seen with the whole bacterial cell system, rather than the isolated lipopolysaccharide system. As seen in the bacterial agglutination system, 2-keto-3-deoxyoctonate effectively competes with the LPS for interacting with the lectin and the inhibition is more marked in the case of the disaccharide which works at approximately 100 times lower concentration (3 to 7 x 10^{-5}M) (Figure 5). It is to be noted from this figure that even if these powerful saccharide inhibitors are used, complete inhibition of interaction has not been achieved, indicating that secondary interactions are involved in stabilizing the lectin-lipopolysaccharide complex.

Our results indicate that carcinoscorpin interacts with the KDO residues of bacterial cells and lipopolysaccharides. The E.coli K12 cells with the O-antigenic chains missing in their LPS and S.minnesota R595 (heptose-less)

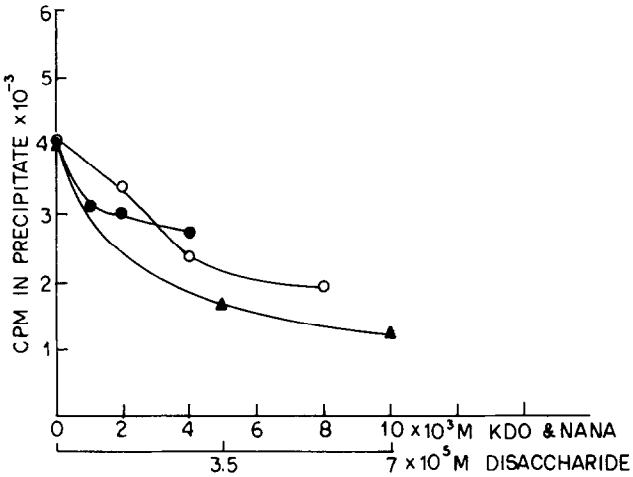

Figure 5. Inhibition of the precipitation of S.minnesota R595 lipopolysaccharide by carcinoscorpin, in the presence of specific saccharides. The conditions of the experiment are as described in the text. 23 µgs of carcinoscorpin, 2.2 x 10^4 cpm of ^{125}I-carcinoscorpin and a fixed amount of S.minnesota LPS (6 nmoles of KDO) were used. 2-keto-3-deoxyoctonate (O—O) ; N-acetylneuraminic acid (●—●) and disaccharide (▲—▲).

cells in which the KDO residues happen to be terminal (like sialic acid in sialoglycoproteins) have proved to be very useful model systems to study their interaction with CSN. Recent work in our laboratory (unpublished results) has shown that KDO is an effective inhibitor of the fetuin-carcinoscorpin interaction as well as carcinoscorpin-mediated rabbit red cell agglutination, thus confirming the specificity of carcinoscorpin towards this 8-carbon acidic sugar. In this connection, it is worth noting the earlier work of Pistole (18,19) who suggested that the Limulus polyphemus serum contains some lectins for interaction with the core and lipid A regions of the lipopolysaccharides. The results presented here using homogeneous carcinoscorpin from the crab Carcinoscorpius rotunda cauda strongly indicate the specific interaction of the lectin with the 2-keto-3-deoxyoctonate residues of lipopolysaccharides. The interaction of CSN with the lipopolysaccharides appears to be in line with the theory that this could be the humoral recognition material in the hemolymph of the crab for its pathogenic bacteria (20,21). But the antibacterial defense function of this lectin, as an opsonin or phagocytosis-promoting material is yet to be demonstrated in vivo. The natural pathogenic microorganism for this crab is not yet known. Recent reports from other laboratories (22,23) and our own work (3) indicates that the sialic acid binding lectins also have antiglucuronyl activity. In this relation it would be particularly interesting to see whether some Gram negative marine bacteria possess KDO and glucuronic acid residues in their lipopolysaccharides. Only future investigations will reveal

the exact dimension of the multispecificity of this unique sialic acid binding lectin.

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