On the multispecificity of carcinoscorpin, the sialic acid binding lectin from the horseshoe crab *Carcinoscorpius rotundacauda*

Recognition of glycerolphosphate in membrane teichoic acids

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Received 19 August 1982

Carcinoscorpin *Sialic acid* *Recognition* *Glycerolphosphate* *Teichoic acid* *Gram-positive bacteria*

1. INTRODUCTION

Our work [1–5] on the structure and function of carcinoscorpin, the sialic acid-specific lectin from the horseshoe crab *Carcinoscorpius rotundacauda,* has indicated that its binding to sialoglycoproteins is dependent not only on the sialic acid content, but also on the type of glycosidic linkage. This lectin recognizes 2-keto-3-deoxyoctonate residues in the lipopolysaccharide of certain Gram-negative bacteria [6]. Apart from this acidic sugar which is very similar to sialic acid, a structurally unrelated acidic sugar such as D-glucuronic acid was also found to be an effective inhibitor of carcinoscorpin–fetuin interaction [3,5]. Looking at the dimension of the multispecificity, we considered the possibility that certain acidic sugar derivatives such as phosphorylated sugars could also interact with this lectin. Of the various phosphorylated sugars and sugar alcohols tested, we came across an interesting specificity of this lectin towards glycerolphosphate which occurs in the membrane teichoic acids of Gram-positive bacteria.

2. MATERIALS AND METHODS

N-Acetylneuraminic acid, β-glycerophosphate, fetuin type III, mannose-6-phosphate, pyruvate, phosphoenol pyruvate, 3-phosphoglyceric acid, *N*-acetylglucosamine, *N*-acetylgalactosamine and adonitol were purchased from Sigma Chemical Co. (St Louis MO). Ribose 5-phosphate, barium salt was from Reanal (Hungary). Carcinoscorpin was purified as in [1,2]. Carrier free ^{125}I was obtained from Bhabha Atomic Research Centre (Bombay). Protein was estimated as in [7] using crystalline bovine serum albumin as standard. *Streptococcus faecalis* (group D, CDC no. SS498) was a kind gift from Dr Grace Koshi (Christian Medical College Hospital, Vellore). Bacteria were grown in 3% brain heart infusion broth at 37°C for 8–10 h with vigorous aeration. The cells were harvested, washed twice and suspended in sterile saline (0.9%).

2.1. *Iodination of fetuin*

This was carried out according to the earlier method [1]. The iodinated protein had spec. act. 6300 cpm/ng. The radioactivity was measured in a Packard Autogamma 5110 counter.

2.2. *Preparation of ribitol-5-phosphate*

Ba^{2+} was removed by treating the suspension of ribose 5-phosphate with a few beads of Dowex 50 (H^+ form). The supernatant was neutralized to pH 7.0 and then subjected to borohydride reduction, as in [8].

2.3. *Binding assay*

The incubation mixture contained in 100 μl final vol. 50 mM Tris–HCl buffer (pH 8.0), 100 mM NaCl, 20 mM $CaCl_2$, 23 μg carcinoscorpin

Published by Elsevier Biomedical Press

00145793/82/0000–0000/$2.75

(0.05 nmol), 5 μg fetuin (0.1 nmol), ^{125}I-labelled fetuin corresponding to 2.4 × 10^4 cpm (spec. act. 6300 cpm/ng) and the various inhibitors at the final concentrations as indicated. The lectin, in the above buffer system was first pre-incubated at room temperature for 30 min with the inhibitor, before adding the mixture containing fetuin and ^{125}I-labelled fetuin. The tubes were incubated for further 60 min at room temperature and then centrifuged at 2000 × *g* for 10 min. The pellet was washed twice with 200 μl 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 20 mM $CaCl_2$ before measuring the radioactivity. ^{125}I-Labelled fetuin alone in the above buffer containing the inhibitor served as the blank control. The extent of inhibition of the carcinoscorpin fetuin complex formation is expressed, keeping the control interaction (without inhibitor) as 100%.

2.4. *Agglutination of bacteria*

The method used was essentially as in [9]. Agglutination was followed at 580 nm, in 1 ml final assay vol. at room temperature (28–30°C). Inhibition of bacterial agglutination was followed in the presence of appropriate concentrations of *N*-acetylneuraminic acid and β-glycerophosphate.

3. RESULTS AND DISCUSSION

The results of the experiments with various phosphorylated sugars and sugar alcohols are given in table 1. Our experiments showed that neither mannose-6-phosphate nor the phosphomannan from *Saccharomyces cerevisiae* inhibited the fetuin–carcinoscorpin interaction. Other phosphates like *p*-nitrophenyl phosphate, β-NADP and pyridoxamine phosphate too did not produce any appreciable inhibition. Most significant inhibition occurred when 5 mM of either α- or β-glycerophosphate was used. The inhibition observed cannot be construed as due to a trapping of Ca^{2+} by β-glycerophosphate, since the binding assay mixture contained 20 mM $CaCl_2$, instead of 10 mM, reported in [5,6]. This finding suggested to us that this lectin might recognize the glycerolphosphate residues occurring in the membrane teichoic acids of Gram-positive bacteria. Control experiments with the other constituents of teichoic acid like glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, glycerol, adonitol and D,L-alanine revealed that none

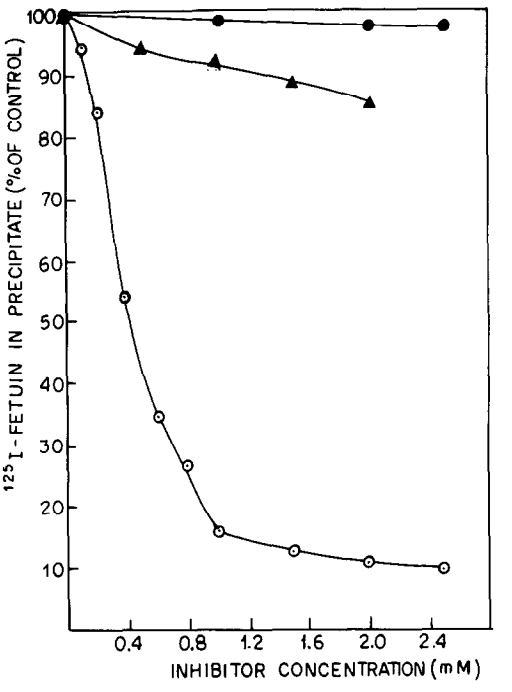

Fig.1. Effect of inhibitor concentration on the fetuin–carcinoscorpin interaction. The details of the experiment are the same as in the text. Each point is a mean of 2 determinations: (○) β-glycerophosphate; (●) glycerol; (▲) ribitol-5-phosphate.

of these compounds produced a significant inhibition like β-glycerophosphate. It is worth noting that *N*-acetylneuraminic acid, to which this lectin is specific, produces less inhibition of the fetuin–lectin interaction at 10 mM than β-glycerophosphate at 5 mM. The significance of the inhibition brought out by phosphoenol pyruvate and 3-phosphoglyceric acid is not very clear. It is possible that this lectin might also recognize pyruvic acid residues occurring in some pneumococcal polysaccharides [10].

We next investigated the effect of inhibitor concentration on the fetuin–carcinoscorpin interaction (fig.1). Marked inhibition was observed with β-glycerophosphate which was 85% at 1 mM final conc. Ribitol-5-phosphate, which occurs on the wall teichoic acids of Gram-positive bacteria produced only a moderate inhibition at 1 mM. Parallel experiments with identical molar concentrations of glycerol revealed no inhibition of the fetuin–carcinoscorpin interaction, indicating that

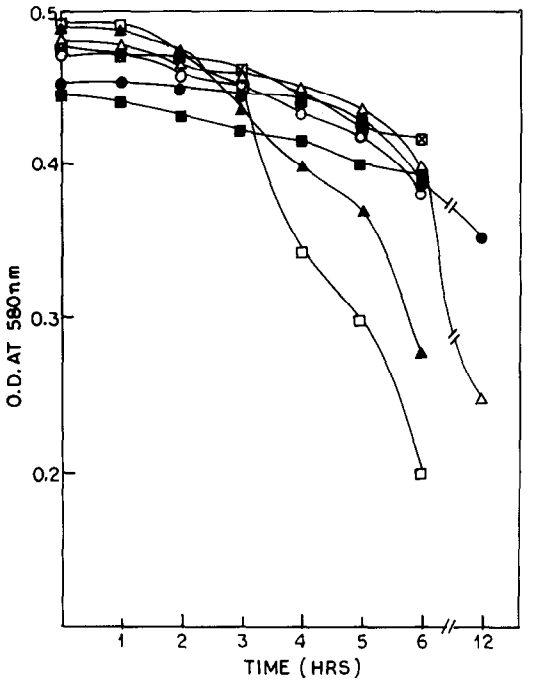

Fig.2. Agglutination of *Streptococcus faecalis* (SS498) group D bacteria by carcinoscorpin and the effect of inhibitors. The incubation mixture contained 50 mM Tris–HCl buffer (pH 8.0), 100 mM NaCl and the bacterial cells enough to give an initial absorbance of ~0.5 (see section 2.4.). Suspension of the cells in buffer without (○), with (●) 10 mM $CaCl_2$ in the absence of the lectin served as controls, to indicate the extent of passive settling of the bacteria. (△) Agglutination of cells in buffer containing 54 μg carcinoscorpin and without Ca^{2+}. At the end of 6 h, $CaCl_2$ was introduced to 10 mM final conc. and the agglutination was followed for further 6 h. (▲) Agglutination of the cells in buffer, containing 10 mM $CaCl_2$ and 58 μg carcinoscorpin. (□) As in (▲), but with 116 μg carcinoscorpin. (■) Agglutination of the cells in buffer containing 10 mM $CaCl_2$, 116 μg carcinoscorpin and 5 mM β-glycerophosphate. (▫) Agglutination of the cells in buffer containing 10 mM $CaCl_2$, 116 μg carcinoscorpin and 10 mM *N*-acetylneuraminic acid.

the inhibition observed with β-glycerophosphate is highly specific. Hence, we chose *Streptococcus faecalis* group D bacteria, in which the hydrophilic teichoic acid chain is known to extend away from the membrane surface and into the wall [11]. Moreover, the lipoteichoic acid with the polyglycerolphosphate residues is established to be the group-specific antigen for *Streptococcus* group D

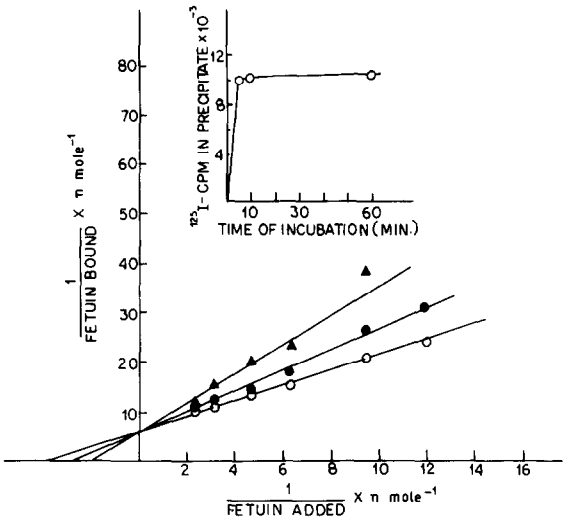

Fig.3. Lineweaver–Burk analysis of the β-glycerophosphate interaction. The details of the experiment are essentially as in the text. The inset shows the effect of incubation time in the fetuin–carcinoscorpin interaction. Since the optimum precipitation of ^{125}I-labelled fetuin was obtained in 5 min, the Lineweaver–Burk analysis was performed by incubating the lectin with fetuin in the presence of the inhibitor for 5 min: (○) no inhibitor; (●) in the presence of 0.4 mM and (▲) 0.8 mM β-glycerophosphate.

Table 1

Effect of various phosphates and teichoic acid constituents on the carcinoscorpin–fetuin interaction

Inhibitor	Final conc. (mM)	% Act.
Control	–	100
Yeast mannan		
(– phosphate)	1.15 (mg/ml)	100
(+ phosphate)	1.15 (mg/ml)	100
D-Mannose-6-phosphate	5	96
Pyruvate	5	85
Phosphoenol pyruvate	5	63
3-Phosphoglyceric acid	5	74
β-Glycerophosphate	5	12
D-Glucose	5	100
N-Acetyl-D-glucosamine	5	100
N-Acetyl-D-galactosamine	5	100
Glycerol	5	96
Adonitol	5	91
D,L-Alanine	5	98
N-Acetylneuraminic acid	10	55

bacteria. The agglutination studies were done as in [6,9] and are shown in fig.2. There is a marked dose-dependent agglutination of the bacteria by carcinoscorpin. This lectin, which requires Ca^{2+} for binding to sialic acid also exhibits the obligatory requirement of Ca^{2+} for binding to the glycerolphosphate residues on the bacteria. This is shown by the observation that in the absence of these ions, the lectin fails to agglutinate the bacterial cells. Marked inhibition of agglutination of the cells occurred when either 5 mM β-glycerophosphate or 10 mM *n*-acetylneuraminic acid was used. The inability of this lectin to agglutinate *Staphylococcus aureus* PS81 cells which possess polyribitolphosphate in their wall teichoic acid was reported in [6]. This finding, when considered along with the strong agglutination of *Streptococcus* group D cells, suggests that carcinoscorpin is specific for glycerolphosphate rather than ribitol-5-phosphate. In this relation it is interesting to note the differential bactericidal activity (resistant *Staphylococcus aureus*) ATCC 25933 compared to susceptible *Enterococcus* group D bacteria) of the *Limulus* amoebocyte lysate, in [12].

The possibility exists from the above studies that β-glycerophosphate can exert its inhibitory activity either by binding to the sialic acid-specific site or to any other site on the carcinoscorpin molecule. Lineweaver–Burk analysis (fig.3) indicates that the effect of β-glycerophosphate in the carcinoscorpin–fetuin interaction is competitive in nature. However, further studies are needed to clarify whether carcinoscorpin recognizes internal or the terminal glycerolphosphate residues in teichoic acids. According to [5], binding profiles of carcinoscorpin with sialoglycoproteins indicated 2 sets of binding sites, one with a high affinity and the other with a low affinity towards sialic acid. While *N*-acetylneuraminic acid and *O*-(*N*-acetylneuraminal)(2→6)2-acetamide-2-deoxy-D-galacticol produced significant quenching of the intrinsic carcinoscorpin fluorescence, the structurally related sugar 2-keto-3-deoxyoctonate failed to do so. We observed that β-glycerophosphate also failed to quench the lectin fluorescence. Whether this lectin molecule is made up of subsites with which 2-keto-3-deoxyoctonate or β-glycerophosphate interact in a manner different from *N*-acetylneuraminic acid is a fundamental problem which emerges from these studies. Preliminary studies with polyglycerolphosphate teichoic acid isolated from *Lactobacillus buchneri* revealed that this teichoic acid effectively competes with fetuin and brings a significant inhibition of the lectin–fetuin complex formation.

These studies demonstrate a new specificity for this sialic acid binding lectin from the horseshoe crab. The biological implications and significance of this finding are many-fold. Our studies suggest that this lectin not only can recognize Gram-negative bacteria, through the 2-keto-3-deoxyoctonate residues, but also Gram-positive bacteria through the glycerolphosphate residues in teichoic acids. It would be interesting to see whether these specific binders are located on the surface of the Gram-negative or Gram-positive bacteria which are pathogenic for this horseshoe crab. Even though the broadspectrum agglutination of bacteria was known for the haemolymph [13] from the horseshoe crab *Limulus polyphemus*, the nature of the multispecificity was not established for limulin. The specific humoral recognition of 2-keto-3-deoxyoctonate in lipopolysaccharides and glycerolphosphate in membrane teichoic acids of bacteria having been established, our results seem to provide a molecular explanation for the primitive host defence machinery [14,15] existing in the haemolymph of these evolutionarily important 'living fossils', known to be around 360 million years old [16], in the absence of immunoglobulins or immunoglobulin-like molecules [17].

Many lectins do interact [9,18,19] with teichoic acids in their capacity to recognize D-glucose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine residues found on these polymers. The difference lies in the finding that while other lectins interact with the sugar residues of glycosylated teichoic acids, carcinoscorpin seems to be the only lectin which can specifically interact with the polyglycerolphosphate residues in these teichoic acids. It is hoped that this lectin will serve as a useful analytical tool for the bacteriologist, to locate the glycerolphosphate teichoic acid on the bacterial cell surface and to differentiate between polyglycerolphosphate and polyribitolphosphate in teichoic acids.

Finally, to our knowledge, this seems to be the first observation of a truly multi-specific nature among all the existing lectins. By definition [20] a lectin is any sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells

and/or precipitates glycoconjugates. The finding that this sialic acid binding lectin also recognizes some polyolphosphates merits an expansion of the above definition.

ACKNOWLEDGEMENTS

We acknowledge with gratitude the generous gifts of teichoic acid samples from Drs J. Baddiley and I.C. Hancock; yeast phosphomannan samples from Dr C.E. Ballou; *Streptococcus* strains from Drs Grace Koshi and C.P. Thangavelu. We also thank Drs Anadi N. Chatterjee, C.V.N. Rao, Manoranjan Singh, Debabrata Roychowdhury, Anuradha Lohia and T.S. Balganesh for their help and advice. S.M. is a Senior Research Fellow and S.S. is a Junior Research Fellow of the Council of Scientific and Industrial Research. This work was supported in part from the grants of the Department of Science and Technology, Government of India.

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