Analysis of Lectin-Dependent Recognition of Desialylated Erythrocytes by Kupffer Cells

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Kupffer cells isolated from the rat liver are able to bind neuraminidase-treated rat erythrocytes via a D-galactose-specific receptor on the cell surface. Binding of desialylated erythrocytes was inhibited by several mono- and oligo-saccharides related to D-galactose, but not by unrelated sugars. However, after phosphorylation at position 6 D-glucose was as good an inhibitor as D-galactose. Two synthetic glycoproteins, D-galactosyl-albumin and, at a higher concentration, D-glucosyl-albumin, strongly inhibit cell contacts. Lectin-mediated binding of desialylated erythrocytes is dependent on the presence of Ca^{2+} ions, but independent of ATP formation and cell motility. It is concluded that binding of desialylated erythrocytes by rat Kupffer cells is mediated by a Ca^{2+} -dependent D-galactosyl/D-glucosyl-recognition system.

It has been repeatedly observed that erythrocytes do not remain in circulation after neuraminidase treatment, but are entrapped in the liver and ingested by Kupffer cells (Durocher *et al.*, 1975; Jancik *et al.*, 1978; Friedrich *et al.*, 1978). Adhesion of desialylated erythrocytes to liver macrophages also occurs *in vitro* (Aminoff *et al.*, 1977), whereas binding of desialylated erythrocytes to isolated hepatocytes could not be demonstrated (which is at variance with our own observations; Kolb *et al.*, 1978). This difference has been discussed elsewhere (Kolb *et al.*, 1978, 1979). We have found that binding of desialylated erythrocytes is due to D-galactose-specific lectin-like receptors present on the surface of Kupffer cells (Kolb & Kolb-Bachofen, 1978) and on the surface of hepatocytes (Kolb *et al.*, 1978). In the present paper we report further characteristics of the Kupffer-cell receptor. The receptor appears to be very similar to the D-galactosyl/D-glucosyl-binding protein on hepatocytes described first by Ashwell & Morell (1974).

Materials and Methods

Animals

Male Osborne–Mendel rats weighing 170–190 g were used. The rats were kindly provided by Dr. L. Herberg, Diabetes Research Institute, University of Düsseldorf.

Kupffer cells

The rat livers were perfused with collagenase (Boehringer, Mannheim, West Germany) (100 mg/150 ml of perfusion medium), in a recirculating system for 12–14 min as described previously (Kolb & Adam, 1976). The perfusion medium contained 137 mM-NaCl, 6.0 mM-KCl, 2.5 mM- Na_2HPO_4 , 2.0 mM- NaH_2PO_4 , 20 mM- NaHCO_3 , 5.0 mM-glucose and 0.028 mM-Phenol Red. The pH was adjusted to 7.4 with 1 M-NaOH. The medium was equilibrated with O_2/CO_2 (19:1) at 38°C.

The liver was transferred into 80–100 ml of ice-cold perfusion medium. The liver capsule was removed and a crude cell suspension prepared by gently teasing the tissue with a pair of forceps. The cell suspension was filtered through nylon mesh (pore size 80 μm). Most of the hepatocytes were removed by repeated centrifugation at 20 g. The Kupffer cells were spun down from the supernatant at 100 g for 4 min and washed twice with modified Eagle's medium (Flow Laboratories, Bonn, W. Germany), buffered with 10 mM-sodium phosphate, pH 7.4, and used immediately for binding tests. The final Kupffer-cell fraction contained 40–60% Kupffer cells. The viability of the cells was more than 95% as determined by Trypan Blue exclusion tests.

At 30 min before perfusion the animals were

injected intravenously with 0.12 ml of Indian ink (Pelikan fount India; Gunther Wagner, Hannover, W. Germany)/100 g body wt., which had been dialysed against 0.9% NaCl.

Erythrocytes

Fresh rat blood was mixed with sodium citrate to prevent clotting and washed three times with modified Eagle's medium. Erythrocytes (10^9 cells/ml) were incubated with 0.1 unit (1 unit $\equiv$ 1 μ mol/min) of neuraminidase (from *Vibrio cholerae*; Behringwerke, Marburg, W. Germany)/ml at 37°C for 30 min and washed three times with modified Eagle's medium.

Binding test and inhibition studies

A portion (50 μ l) of freshly isolated Kupffer cells (2×10^6 cells/ml) was mixed with 50 μ l of erythrocytes (10^8 cells/ml) in round-bottomed tubes and incubated for 5 min on ice. The tubes were centrifuged at 100 g for 5 min and incubated at 4°C for 1 h. The cells were resuspended by shaking the tubes by hand and counted in a haemocytometer by light-microscopy. Cells that had ingested ink particles were regarded as Kupffer cells. Kupffer cells binding with three or more erythrocytes were considered as rosette-forming cells. Each determination was done in duplicate, about 100 Kupffer cells being examined per sample.

Carbohydrate inhibition studies were performed by preincubating Kupffer cells with sugars or glycoproteins for 5 min at 0°C. Galactonolactone, stachyose, D-(+)-raffinose, 2-deoxy-D-galactose, lactulose, D-glucose 6-phosphate, D-mannose 6-phosphate and D-galactose 6-phosphate were purchased from Sigma (München, W. Germany). D-Glucose, D-mannose, D-fucose, D-galactose and α -D-melibiose came from Serva (Heidelberg, W. Germany). N-Acetyl-D-galactosamine was from Roth (Karlsruhe, W. Germany) and L-fucose from Calbiochem (San Diego, CA, U.S.A.). Orosomucoid, desialylated orosomucoid, D-glucosyl-(bovine serum albumin) and D-galactosyl-(bovine serum albumin) were gifts from Dr. G. Ashwell (National Institutes of Health, Bethesda, MD, U.S.A.) and Dr. Y. C. Lee (Johns Hopkins University, Baltimore, MD, U.S.A.) respectively. Preincubation of Kupffer cells with NaN_3 , colchicine, cytochalasin B (Serva) and Pronase (Calbiochem) was performed for 30 min at 37°C.

Inhibition studies with anti-(rat immunoglobulin) antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) were done as follows. γ -Globulin fractions were prepared by precipitation with Na_2SO_4 and absorbed twice with a mixture of untreated and neuraminidase-treated rat erythrocytes. The activity and specificity of the antibody

fractions were tested with rat lymphocytes from thymus and spleen in the immunofluorescence test.

Electron microscopy

Binding assays were performed as described above. The pellet of Kupffer cells and erythrocytes was fixed in 100 mM-cacodylate/HCl buffer containing 150 mM-saccharose, 2 mM- CaCl_2 and 12.5 ml of glutaraldehyde (Serva)/litre and postfixed in the same buffer with 10 mg of OsO_4 /litre. Samples were dehydrated with ethanol and embedded in epoxy resin as described by Spurr (1969). Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Results

Specificity of recognition of desialylated erythrocytes by Kupffer cells

Several monosaccharides, oligosaccharides and glycoproteins have been screened for their ability to interfere with the binding of neuraminidase-treated rat erythrocytes to rat Kupffer cells *in vitro*. Of all the saccharides tested, only those related in structure to D-galactose were inhibitory (Table 1). This supports our previous observation, based on a study with six sugars (Kolb & Kolb-Bachofen, 1978).

The most effective monosaccharide was N-acetyl-D-galactosamine. Oligosaccharides with D-galactose in the terminal non-reducing position also reacted with the Kupffer-cell lectin. Both the α - and β -conformations of D-galactosyl residues were found to be effective (Table 1). None of the oligosaccharides tested was a better inhibitor than N-acetylgalactosamine.

Desialylated orosomucoid has been shown previously to interact with the D-galactose-specific lectin on hepatocytes described by Lunney & Ashwell (1976). It similarly reacts with the Kupffer-cell receptor (Table 1). We have also tested whether phosphorylated saccharides are better inhibitors than normal sugars, since such observations have been made for a mannose-specific lectin present on liver epithelial cells and fibroblasts (Ullrich *et al.*, 1978). No such effect was found for the recognition of D-galactose and D-mannose. In contrast, D-glucose phosphorylated in position 6 reacted with the Kupffer-cell receptor to a similar degree as did D-galactose, whereas glucose itself scarcely inhibited lectin-dependent cell contacts (Table 2).

The reactivity of the lectin towards both D-galactose and D-glucose moieties became more apparent when synthetic glycoproteins were used. In Table 3 it is shown that bovine serum albumin substituted with either D-glucose or D-galactose interfered with binding of desialylated erythrocytes to Kupffer cells.

Table 1. *Specificity of recognition of desialylated erythrocytes by Kupffer cells*

Kupffer cells were preincubated with the inhibitor for 5 min at 0°C before the usual binding assay. The data are the averages ($\pm$ S.D.) from 4–12 experiments. Binding of desialylated erythrocytes to Kupffer cells was regarded as positive when three or more erythrocytes were bound. In these experiments $76 \pm 10\%$ Kupffer cells bound desialylated erythrocytes in the absence of inhibitors [average ($\pm$ S.D.) of seven experiments].

Inhibitor	Concn. necessary for 50% inhibition (mM)
Monosaccharides	
D-Mannose	>50
D-Glucose	>50
N-Acetyl-D-glucosamine	>50
Galactonolactone	>25
L-Fucose*	7 ($\pm$ 2.8)
D-Galactose*	4.4 ($\pm$ 2.7)
D-Fucose*	3.8 ($\pm$ 0.9)
Deoxygalactose	3.2 ($\pm$ 1.0)
N-Acetyl-D-galactosamine*	0.7 ($\pm$ 0.3)
Oligosaccharides	
Stachyose [α -Gal(1 $\rightarrow$ 6) α -Gal(1 $\rightarrow$ 6) α -Glc(1 $\rightarrow$ 2) β -Fru]	>25
Raffinose [α -Gal(1 $\rightarrow$ 6) α -Glc(1 $\rightarrow$ 2) β -Fru]	9.4 ($\pm$ 7.0)
Lactose [β -Gal(1 $\rightarrow$ 4)Glc]	2.5 ($\pm$ 1.3)
Melibiose [α -Gal(1 $\rightarrow$ 6)Glc]	2.3 ($\pm$ 1.5)
Lactulose [β -Gal(1 $\rightarrow$ 4)Fru]	1.1 ($\pm$ 0.3)
Glycoproteins	
Orosomucoid	>1
Desialylated orosomucoid	0.03 $\pm$ 0.01

* In a previous preliminary report (Kolb & Kolb-Bachofen, 1978) approximately five times higher saccharide concentrations were needed for 50% inhibition of cell contacts. This difference is probably due to the use of a different rat strain.

Table 2. *Effect of phosphorylated monosaccharides on recognition of desialylated erythrocytes by Kupffer cells*
Kupffer cells were preincubated with the saccharide for 5 min at 0°C before the usual binding assay. The data are the averages ($\pm$ S.D.) from three experiments.

Saccharide	Concn. necessary for 50% inhibition (mM)
D-Galactose	4.4 ($\pm$ 2.7)
D-Galactose 6-phosphate	5.7 ($\pm$ 4.3)
D-Glucose	>25
D-Glucose 6-phosphate	7.1 ($\pm$ 4.7)
D-Mannose	>25
D-Mannose 6-phosphate	25 ($\pm$ 14)

Table 3. *Affinity of the Kupffer-cell receptor for D-galactose and D-glucose covalently bound to bovine serum albumin*

Kupffer cells were preincubated with the glycoprotein for 5 min at 0°C before the usual binding assay. Glucosyl- and galactosyl-BSA are synthetic glycoproteins with 35 mol of D-glucose or 37 mol of D-galactose respectively per mol of bovine serum albumin. Both were kindly provided by Dr. Y. C. Lee (Johns Hopkins University, Baltimore). The data are the averages ($\pm$ S.D.) from three experiments.

Glycoprotein	Concn. necessary for 50% inhibition (μ M)
Glucosyl-BSA	0.7 $\pm$ 0.3
Galactosyl-BSA	0.06 $\pm$ 0.02
BSA	>100

Physical characteristics of cell contacts between Kupffer cells and desialylated erythrocytes

Kupffer cells were incubated with NaN_3 , an inhibitor of ATP formation, at 37°C for 30 min. The rate of rosette formation was only slightly decreased (Table 4), indicating that receptor-mediated cell contact is independent of energy metabolism. This is

in accord with the observation that inhibitors of cell motility (cytochalasin B, colchicine) have no effect on erythrocyte binding (Table 4). After the addition of EDTA and EGTA cell adhesion is almost totally abolished, demonstrating that receptor function depends on the presence of Ca^{2+} ions. Treatment of

Table 4. *Physical characteristics of cell contacts between Kupffer cells and desialylated erythrocytes*

The number of Kupffer cells binding three or more desialylated erythrocytes was determined. Kupffer cells were preincubated with the inhibitor for 30 min at 37°C. The data are the averages ($\pm$ S.D.) from three to five experiments. Goat and rabbit anti-(rat immunoglobulin) sera were used in 1:20 dilution. This concentration was sufficient to reach saturation levels for the reaction of the antisera with membrane-bound immunoglobulins as judged from immunofluorescence studies.

Inhibitor	Concn.	Inhibition (%)
NaN ₃	10 mM	15 ($\pm$ 3.8)
Cytochalasin B	0.04 mM	0
Colchicine	0.25 mM	0
EDTA	2 mM	95 ($\pm$ 3.2)
EGTA	2 mM	98 ($\pm$ 1.5)
Pronase	1.5 mg/litre	95 ($\pm$ 2)
Goat antiserum	1:20	<10
Rabbit antiserum	1:20	<10

Kupffer cells with bacterial proteinases (Pronase) led to a complete loss of binding capacity (Table 4).

The recognition of desialylated erythrocytes by Kupffer cells could not be blocked by an excess of anti-(rat γ -globulin) antibodies, indicating that natural antibodies bound on the liver macrophage cell surface are not involved (Table 4). Binding assays were performed in the presence of anti-rat antibodies, which should mask all antibodies on the macrophage surface. We used goat and rabbit anti-(rat immunoglobulin G) antibodies at a concentration which reached saturation levels in the immunofluorescence activity test. Inhibition of the binding was less than 10% in the presence of antibodies, as tested in three experiments.

Ultrastructural analysis of the cell-cell contact

Analysis of the binding between desialylated erythrocytes and Kupffer cells by electron microscopy shows broad contact zones (Plate 1). The pseudopodia of the macrophage surround the bound erythrocyte. This may be regarded as an initial step in phagocytosis. The two plasma membranes of both cells show no fusion configurations. Compared with the binding of desialylated erythrocytes to hepatocytes (Kolb *et al.*, 1978) there is a marked difference; hepatocytes form cell contacts with erythrocytes only at the tips of their microvilli.

Discussion

In the experiments described here we show that the D-galactose-specific receptor on Kupffer cells has

characteristics very similar to those of the corresponding lectin on hepatocytes, described first by Ashwell & Morell (1974). The Kupffer-cell receptor binds D-galactose or related monosaccharides; N-acetylgalactosamine is bound with higher affinity than D-galactose. The same observations have been made for the hepatic binding protein (Stockert *et al.*, 1977). Both receptors recognize oligosaccharides with a D-galactose residue at the non-reducing terminal.

Desialylated orosomucoid is recognized by the Kupffer-cell receptor as well as by the hepatocyte lectin (Lunney & Ashwell, 1976). Desialylated orosomucoid is bound with 30–40 times greater avidity than the most effective mono- and oligosaccharides in our system. This may be an indication that the receptor recognizes a sequence of more than one saccharide. The Kupffer-cell lectin recognizes not only D-galactose but also D-glucose. Evidence is given by experiments where D-galactose as well as D-glucose coupled to bovine serum albumin strongly inhibited receptor activity. The same is true for the hepatic binding protein (Stowell & Lee, 1978). In contrast with these findings with glycoproteins, we did not observe reactivity of the hepatocyte or Kupffer-cell receptor with D-glucose monosaccharide (Kolb *et al.*, 1978; Kolb & Kolb-Bachofen, 1978). It is interesting, however, that D-glucose is bound with high affinity to the Kupffer-cell lectin after phosphorylation in position 6. A similar preferential binding of phosphorylated saccharides has been described for D-mannose-specific receptors on fibroblasts and liver epithelial cells (Kaplan *et al.*, 1977; Sando & Neufeld, 1977; Ullrich *et al.*, 1978). The reactivity of the hepatocyte binding protein towards phosphorylated glucose has not yet been determined. The Kupffer-cell receptor function depends on the presence of Ca²⁺ ions. This property is again shared by the hepatic lectin (Ashwell & Morell, 1974). These findings lead us to suggest that the receptor on Kupffer cells is identical with the receptor on hepatocytes.

We have shown that the Kupffer-cell receptor acts as a lectin mediating cell contacts with desialylated erythrocytes under conditions prohibiting enzymic activity. This does not exclude an additional enzyme function of the receptor. It has been shown that a galactose oxidase in its inactive form could function as a lectin (Horejsi, 1979).

The function of the Kupffer-cell receptor is not known. Ashwell & Morell (1974) assumed the control of glycoprotein turnover to be the function of the hepatocyte lectin. Kupffer cells, on the other hand, are known to recognize and phagocytose senescent erythrocytes. The receptor for D-galactose may facilitate the adhesion of aged erythrocytes to the macrophage. There is, however, no firm evidence for the exposure of D-galactosyl or D-glucosyl

EXPLANATION OF PLATE 1

Ultrastructure of cell contacts between Kupffer cells and desialylated erythrocytes

Two erythrocytes (ery) are bound by the Kupffer cell (kuc). The macrophage pseudopodia (pp) are surrounding the erythrocytes, indicating the beginning of phagocytosis.

residues on senescent erythrocytes (Baxter & Beeley, 1975; Marikovsky *et al.*, 1976). A lectin-like receptor on macrophages may also have an immune function. D-Galactose residues are known to be expressed on a number of pathogenic microbes, as well as on malignant or on virus-infected cells (Springer, 1971; Hakomori, 1975; Martin, 1975; Warren *et al.*, 1978). The interaction between Kupffer cells and senescent erythrocytes and tumour cells requires study.

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