Interaction of bilirubin with human erythrocyte membranes

Bilirubin binding to neuraminidase- and phospholipase-treated membranes

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Saturable bilirubin binding to human erythrocyte membranes was measured before and after digestion with neuraminidase and phospholipases. Neuraminidase-treated erythrocyte membranes did not show any change in their binding properties, indicating that gangliosides could be excluded as candidates for saturable bilirubin-binding sites on erythrocyte membranes. Although bilirubin-binding properties of the membranes did not change after phospholipase D digestion, either, phospholipase C treatment greatly enhanced bilirubin binding. Thus it is suggested that a negatively charged phosphoric acid moiety of phospholipids on the membrane surface may play a role to prevent a large amount of bilirubin from binding to the membranes. Further saturable bilirubin binding to inside-out sealed erythrocyte membrane vesicles showed values comparable with those of the right-side-out sealed membranes, suggesting that the bilirubin-binding sites may be distributed on both outer and inner surfaces of the membranes, or may exist in the membranes where bilirubin may be accessible from either side.

INTRODUCTION

Although it is well known that bilirubin exhibits multi-site toxicity towards the metabolism of living cells (Karp, 1979; Kashiwamata *et al.*, 1984), understanding of the pathway(s) through which bilirubin enters the inside of cells is still scarce. As the first step to explore the mechanism, we have developed a method to assay the saturable bilirubin-binding sites on human erythrocyte membranes and reported some binding properties of the membranes (Sato & Kashiwamata, 1983). The binding sites were considered not to be composed of proteins, because the binding capacities were not decreased, but were increased, by tryptic digestion and heat treatment of the membranes. Affinity labelling of the membrane proteins by bilirubin could not identify any specific bilirubin-binding protein (Sato & Kashiwamata, 1983).

Since gangliosides (Kahán *et al.*, 1968; Weil & Menkes, 1975) and/or polar head groups of phospholipids (Nagaoka & Cowger, 1978; Brodersen, 1979) have been assumed to be bilirubin-binding sites on the surface of plasma membranes, the effects of neuraminidase and phospholipases on bilirubin binding to human erythrocyte membranes were investigated in the present study. Our present results do not support such hypotheses as mentioned above. On the contrary, it is suggested that negatively charged polar head groups of phospholipid molecules may prevent a large amount of bilirubin from binding to the membranes.

MATERIALS AND METHODS

Chemicals and enzymes

The products used were neuraminidase from *Clostridium perfringens* (Boehringer), phospholipase C from *Bacillus cereus* (Boehringer) and *Clostridium welchii* (Sigma), phospholipase D from cabbage (Boehringer), β -N-acetylhexosaminidase from *Turbo cornutus* (Seikagaku Kogyo, Japan), β -galactosidase from *Escherichia coli* (Worthington), dipalmitoyl-DL- α -phosphatidylcholine (Sigma, grade I), L- α -phosphatidic acid (sodium salt)

from egg lecithin (Sigma, grade I) and sphingomyelin from bovine brain (Sigma, type I). [3 H]Bilirubin was purchased from Institut National des Radioéléments (France). Other reagents were of the highest grade commercially available.

Preparation of erythrocyte membranes and bilirubin solution

Fresh normal adult erythrocytes were obtained as described previously (Sato & Kashiwamata, 1983). Erythrocyte membranes were prepared by lysis in 10 mM-Tris/HCl buffer, pH 7.4, by the method of Okuda *et al.* (1972). Preparation of inside-out sealed vesicles and right-side-out sealed membranes, and an enzymic check for the sidedness by using acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase, were performed as described by Steck (1973). Bilirubin and [3 H]bilirubin (27.3 Ci/mol) solutions were prepared in the same manner as reported previously (Sato & Kashiwamata, 1983).

Assay of binding of [3 H]bilirubin to human erythrocyte membranes

[3 H]Bilirubin binding to erythrocyte membranes was determined essentially as described in our previous paper (Sato & Kashiwamata, 1983) with minor modifications. For assays with inside-out sealed membrane vesicles and right-side-out sealed membranes, incubation time was decreased from 30 to 10 min, since incubation at 37 °C gradually disrupted the sealing. The activity of acetylcholinesterase (a marker enzyme for the outer surface of the membranes) in the inside-out vesicles increased from 13 (zero time) to 29 % of that in the Triton X-100-treated completely unsealed vesicles by 10 min of incubation in both the total and the non-saturable bilirubin-binding assays. The activity of glyceraldehyde-3-phosphate dehydrogenase (a marker enzyme for the inner surface) in the sealed right-side-out ghosts was 13 and 10 % of that in the Triton X-100-treated unsealed membranes after incubation for 10 min in the total and non-saturable binding assay respectively.

Enzymic digestion of erythrocyte membranes

Phospholipase C (*B. cereus*) treatment: 1.5 ml of the ghost suspension (5 mg of protein/ml) was incubated for 15 min at 37 °C in the reaction mixture containing 1 ml of 100 mM-CaCl₂, 8.26 ml of 50 mM-Tris/HCl buffer, pH 7.4, and 0.75 ml of the enzyme solution (3.75 units). Phospholipase C (*C. welchii*) treatment: 1 ml of the ghost suspension was incubated for 30 min at 37 °C with 3.93 ml of 0.1 M-Tris/HCl buffer, pH 7.4, 50 µl of 1 M-CaCl₂, and 0.25 ml of the enzyme solution (10 mg/ml). Phospholipase D digestion: 0.5 ml of the ghost suspension was digested for 39 min at 37 °C in the reaction mixture containing 1.3 ml of 0.1 M-Tris/HCl buffer, pH 7.4, and 0.2 ml of the enzyme solution (2.5 units). Neuraminidase treatment: 2.5 ml of the ghost suspension was digested for 30 min at 37 °C in 0.1 M-Tris/HCl buffer, pH 7.4, containing 2.1 units of the enzyme. Other enzymic treatment: gangliosides of the membranes were digested for 2 h at 37 °C in the reaction mixture containing 1.6 ml of the ghost suspension, 0.5 ml of 1.5 mM-MgCl₂, 1.5 ml of 0.1 M-acetate buffer, pH 5.6, and the enzyme solution (0.1 unit of neuraminidase, 0.05 unit of β -N-acetylhexosaminidase and 47 units of β -galactosidase) (Gatt, 1969). The control experiments were all carried out without the enzymes.

Extraction and preparation of membrane lipids

Gangliosides of human erythrocyte membranes were extracted by the method of Suzuki (1965). T.l.c. of the gangliosides was performed by the methods of Kundu (1981). Total membrane lipids were extracted by the method of Folch *et al.* (1951). The lipid extracts were stored at -20 °C and sonicated just before use. Purified lipids such as oleic acid, monostearin, phosphatidic acid and phosphatidylcholine were dissolved in chloroform and, after evaporation, suspended in 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA by sonication.

Other methods

Protein concentration was determined by the biuret method (Layne, 1957) or the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. P_i in the membranes was determined by the method of Bartlett (1959). Assays for choline and sialic acid were performed by the methods of Kates & Sastry (1969) and Warren (1959) respectively. Radioactivity of [³H]bilirubin was determined in a Beckman LS-233 liquid-scintillation counter. Absorption spectra were recorded with a Shimadzu UV-210A double-beam spectrophotometer.

RESULTS

Saturable binding of [³H]bilirubin to neuraminidase-treated or inside-out sealed erythrocyte membrane vesicles

Neuraminidase treatment released 88% of sialic acid in the human erythrocyte membranes and 82% of that in their gangliosides (Table 1), and almost all of the ganglioside bands detected with resorcinol on t.l.c. plates disappeared or were markedly decreased by neuraminidase treatment (results not shown). Fig. 1 shows the effect of neuraminidase treatment of human erythrocyte membranes on [³H]bilirubin binding. The enzymic digestion did not significantly decrease the binding of

Table 1. Effect of neuraminidase treatment on sialic acid content in human erythrocyte membranes

Values are means $\pm$ S.D.

Sample	No. of experiments	Sialic acid (nmol/mg of protein in original membranes)
Erythrocyte membranes		
Control	5	50.9 $\pm$ 6.0
Digested	5	6.2 $\pm$ 2.9
Gangliosides extracted from the membranes		
Control	7	1.48 $\pm$ 0.29
Digested	8	0.26 $\pm$ 0.17

bilirubin. The apparent dissociation constant (K_d) and maximum binding (B_{max}) for bilirubin of the treated membranes (4.1 μ M and 1.6 nmol/mg of protein respectively) were almost the same values as those of the control (3.0 μ M and 1.3 nmol/mg of protein). A complete digestion of gangliosides to ceramide glucoside by a mixture of neuraminidase, β -N-acetylhexosaminidase and β -galactosidase increased the binding by as much as 60% (averages of two determinations). Addition of sialic acid (final concn. 1 mM) to the reaction mixture for the binding assay produced no change either in the amount of bound bilirubin or in the absorption spectrum of bilirubin. Neither D-glucose nor N-acetyl-D-galactosamine at 1 mM had any effect on the bilirubin spectrum.

Since gangliosides are known to locate on the outer surface of the membranes (Rothman & Lenard, 1977), the binding to bilirubin on the inside-out sealed membranes was studied (Table 2). Although the K_d and B_{max} for bilirubin of the inside-out sealed vesicles were 30 and 20% less than those of the right-side-out membranes, respectively, the saturable process of bilirubin binding did not disappear after the treatment. The phospholipid/bilirubin molar ratios of right-side-out and inside-out sealed membranes were 255 and 314 respectively.

Fig. 1. Effect of neuraminidase digestion of erythrocyte membranes on the saturable binding of [³H]bilirubin

O, Neuraminidase-digested membranes; ●, control. Each point is the mean $\pm$ S.D. for three separate determinations.

Table 2. Binding of [³H]bilirubin to inside-out and right-side-out sealed human erythrocyte membrane vesicles

Values for molar ratios are calculated from reported values of 306 μg of phospholipid/mg of erythrocyte membrane protein (Sato, 1972) and 750 as an average M_r for phospholipid. Each value is the mean $\pm$ S.D. for five separate determinations.

Sample	Dissociation constant, K_d (μM)	Maximum binding, B_{max} (nmol/mg of protein)	Molar ratio of phospholipid/bilirubin
Right-side-out membranes	6.0 ± 2.4	1.6 ± 0.48	255
Inside-out membrane vesicles	4.2 ± 1.3	1.3 ± 0.14	314

Digestions of erythrocyte membranes by phospholipases

Phospholipase C treatment. Fig. 2 shows the effect of phospholipase from *B. cereus*, which releases the polar head groups of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine, and produces diacylglycerols (Zwaal *et al.*, 1973). The treatment released 74% of the membrane P_i (digested, 0.34 μmol of P_i /mg of ghost protein; control, 1.31 μmol of P_i /mg of ghost protein). The reaction mixture for the binding assay become turbid during incubation, and added bilirubin precipitated with the digested membranes. Hence, the saturable bilirubin-binding assay (Sato & Kashiwamata, 1983) could not be performed. Even less than 5% release of membrane P_i by the enzymic digestion greatly enhanced the bilirubin binding, resulting in precipitation of bilirubin-membrane complexes. Washing of the digested membranes by 0.1 M-Tris/HCl buffer, pH 7.4, containing 6 mM-EGTA did not decrease the enhanced non-saturable binding. Phospholipase C from *Cl. welchii* is reported to be able to degrade sphingomyelin as well as phosphatidylcholine (Zwaal *et al.*, 1973). Treatment with this phospholipase C of the membranes gave the same results as that by the enzyme from *B. cereus* (results not shown).

Phospholipase D treatment. Phospholipase D digestion is known to remove the polar head groups of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, and to form phosphatidic acid (Zwaal *et al.*, 1973). The enzymic treatment of the membranes released 19.6 μg of choline/mg of protein, which corresponded to the degradation of 18% of membrane phosphatidylcholine. Bilirubin binding, however, was not so markedly increased by the treatment (Fig. 3), a finding quite different from that with phospholipase C. The K_d and B_{max} for bilirubin of the digested membranes were, respectively, 4.4 μM and 1.9 nmol/mg of protein, and those of the control membranes were 4.6 μM and 1.5 nmol/mg of protein.

Effects of phospholipids and their constituents on the absorption spectrum of bilirubin

Addition of erythrocyte membranes to bilirubin solution affected the absorption spectrum of bilirubin; a

Fig. 2. Effect of digestion by phospholipase C (*B. cereus*) of erythrocyte membranes on the binding of [³H]bilirubin

●, [³H]Bilirubin; ▲, [³H]bilirubin + 100 μM non-radioactive bilirubin; ○, [³H]bilirubin - ([³H]bilirubin + 100 μM non-radioactive bilirubin). (a) Phospholipase C-digested membranes. (b) Control. Because of precipitation of added bilirubin, the binding assay could not be performed. A typical result is shown. The detailed assay method was described in Sato & Kashiwamata (1983).

Fig. 3. Effect of phospholipase D digestion of erythrocyte membranes on the binding of [³H]bilirubin

○, Phospholipase-D-treated; ●, control. Each value is the mean $\pm$ S.D. for three separate determinations.

peak of bilirubin at 440 nm shifted to 445–450 nm, and a shoulder appeared at 490 nm (Fig. 4a). Extracted lipids from the membranes, phosphatidylcholine, sphingomyelin, oleic acid, monostearin and phosphatidic acid also produced a shift of the 440 nm peak of bilirubin to a broad 450 nm peak with a shoulder at 490 nm (Figs. 4b and 4c; only the results with extracted lipids and oleic acid are shown). Addition of choline chloride, serine and ethanolamine, represented as phospholipid head groups, however, produced no appreciable changes of the bilirubin spectrum (Fig. 4d; only the results with choline

Fig. 4. Effects of erythrocyte membranes, extracted lipids, oleic acid and choline chloride on the absorption spectrum of bilirubin

The basal medium consisted of 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA (total volume 2 ml). (a) Erythrocyte membranes (0.1 ml; 5 mg of ghost protein/ml): curve a, 20 μ M bilirubin alone; curve b, bilirubin and membranes; curve c, membranes alone. (b) Extracted lipids (0.1 ml; 0.078 μ mol of P_i /ml of 50 mM-Tris/acetate buffer, pH 8.0, containing 1 mM-EDTA): curve a, 20 μ M-bilirubin alone; curve b, 20 μ M-bilirubin and extracted lipids; curve c, extracted lipids alone. (c) Oleic acid (final concn. 4 mM): curve a, 20 μ M-bilirubin alone; curve b, bilirubin and oleic acid; curve c, oleic acid alone. (d) Choline chloride (final concn. 4 mM): curve a, 20 μ M-bilirubin alone; curve b, bilirubin and choline chloride; curve c, choline chloride alone.

chloride are shown), and these observations were also supported by the facts that an excess of these substances (4 mM each with 6.6 μ M-[3 H]bilirubin) did not influence the saturable bilirubin binding (results not shown). Neither phosphocholine chloride (4 mM) nor glycerol (4 mM) had any effect on the spectrum of bilirubin.

DISCUSSION

In our previous paper, effects of tryptic digestion and heat treatment on human erythrocyte membranes were reported (Sato & Kashiwamata, 1983). These treatments did not decrease, but increased, the saturable bilirubin-

binding capacity on the membranes, suggesting that membrane proteins may not be the entity constituting the saturable binding sites. Kahán *et al.* (1968) and Weil & Menkes (1975) proposed gangliosides as bilirubin-binding substances on the surface of plasma membranes, on the basis of studies by t.l.c. and absorption spectra of the bilirubin-ganglioside complex. However, in our present experiments, the saturable bilirubin binding was not diminished by the neuraminidase treatment (Fig. 1). In addition, in spite of the presence of gangliosides on the outer surface of the membranes (Rothman & Lenard, 1977), the binding capacity of inside-out sealed membrane vesicles was comparable with that of right-side-out sealed vesicles (Table 2). These results may not support their idea that the binding sites are composed of gangliosides. Our data also suggest that the saturable bilirubin-binding sites may be localized in the membranes, where bilirubin molecules are accessible from either side or distribute on both the outer and the inner surface of the membranes.

Nagaoka & Cowger (1978) and Brodersen (1979) have suggested that the bilirubin-binding sites on the membranes are composed of the polar head-groups of phospholipids. Phospholipase D digestion removes choline, serine and ethanolamine from phospholipids (Zwaal *et al.*, 1973) and produces phosphatidic acid. Such treatment, however, did not influence the bilirubin-binding properties of the membranes (Fig. 3). Digestion of the membranes by phospholipase C with and without sphingomyelinase activity did not decrease, but increased, the bilirubin binding remarkably (Fig. 2). Removal of only a few per cent of the membrane phosphorus in phospholipids greatly enhanced the binding. Furthermore, addition of an excess of substances composing the polar head-groups of phospholipids did not inhibit the saturable bilirubin binding. These observations indicate that the saturable bilirubin-binding sites do not involve the polar head-groups of phospholipids.

The reason why phospholipase C digestion of the membranes greatly enhanced the bilirubin binding may be as follows. The release of polar head-groups from the membranes and the subsequent exposure of non-polar diacylglycerols on the surface of the membranes may potentiate a large amount of non-specific binding of bilirubin to the fatty acid moiety of diacylglycerols. Since bilirubin binds the fatty acid moiety of phospholipids (Fig. 4) and, at physiological pH, purified phospholipids are known to catalyse precipitation of bilirubin (Brodersen, 1979; Eriksen *et al.*, 1981), this reaction will produce an extraordinarily large amount of bilirubin on the digested membranes. The situation seems to be different for phospholipase D digestion. Phosphatidic acid formed in the membranes by the action of phospholipase D has a phosphate group, whose negative charges will bring about electrostatic repulsion against the carboxy groups of bilirubin. Thus it is likely that the negatively charged phosphoric acid moiety of phospholipids may play a role to prevent a large amount of bilirubin from binding to the membranes.

The spectral analyses of bilirubin in the presence of various substances (Fig. 4) strongly suggest that bilirubin preferentially binds to acyl residues rather than the polar head-groups of phospholipids. Nagaoka & Cowger (1978) reported the K_d values for bilirubin of several phospholipids (0.27–6.3 μM), which are comparable with that obtained from human erythrocyte membranes (Sato

& Kashiwamata, 1983). Although no clear evidence has been yet obtained, it may be possible that the saturable bilirubin-binding sites occupy particular positions on the membranes where the hydrophobic part of phospholipids or other lipophilic substance(s) are exposed. Ca^{2+} ions are known to bind to phosphatidylserine to form Ca^{2+} -chelated aggregates, and to induce solidification of membranes and lateral separation of membrane phospholipids (Ohnishi & Ito, 1974; Ito *et al.*, 1975). Addition of 1 mM- CaCl_2 to the assay medium significantly enhanced the total and non-saturable bilirubin binding, by as much as 38 and 42% of the respective control values. However, the saturable binding showed only a slight, non-significant, increase (H. Sato, unpublished work).

In conclusion, although the polar head-groups of phospholipids have been suggested to be binding sites of bilirubin on the plasma membranes, our results indicate that they are probably not the saturable bilirubin-binding sites, but, in contrast with the above-mentioned idea, a negatively charged phosphoric acid moiety of phospholipids prevents a large amount of bilirubin from binding to the membranes. No evidence for the binding of bilirubin to membrane gangliosides could be obtained. The experiments with use of inside-out and right-side-out sealed membranes suggested that the binding sites may be in an inner part of the membranes where bilirubin can reach from either side, or that the sites exist on both the outer and the inner surface of the membrane.

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