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The Effect of Bilirubin on the Surface Charge and Aggregation Tendency of Platelets in Cord Blood

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Abstract. Unbound unconjugated bilirubin markedly increased the negative electrophoretic mobility of washed platelets from cord blood, even at concentration as low as 1.5 mg/dl. The increase in negativity could be abolished by washing the platelets. Aggregation of platelets was observed in parallel with the increase in negativity. These actions of bilirubin required calcium ions, which could not be replaced by magnesium ions. The results suggest attachment of the negative bilirubin molecules onto the platelet surface, probably through calcium ions, leading to platelet aggregation.

Plasma components inhibited the actions of bilirubin on platelets up to 19.5 mg/dl bilirubin concentration.

The effect of bilirubin on platelets was also investigated in the presence of albumin. When the saturation of albumin with bilirubin was exceeded, platelet negativity increased. However, the fraction of free bilirubin exceeding the albumin saturation point has not the same effect as truly free bilirubin at that concentration in the complete absence of albumin. The results indicate that albumin molecules loosly adsorbed onto the surface may protect the platelets against the attachment of free bilirubin. This protection, however, might be impaired by acidosis, which is frequently combined with hyperbilirubinaemia in the sick newborn. It is suggested that bilirubin could contribute to the haemostatic abnormalities in sick babies by acting on the platelet surface.

Key words: Newborn infant – Blood coagulation – Hyperbilirubinaemia – Platelet surface – Cell electrophoresis.

Introduction

The newborn infant, particularly the sick baby, appears to be susceptible to bleeding or thrombotic complications (Hathaway, 1975). Experimental obser-

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vations show that hyperbilirubinaemia plays an important role in the pathogenesis of haemostatic abnormalities (Metze, 1977). Rozdilsky (1961) found that infusion of bilirubin into animals caused not only kernicterus but also haemorrhage in the intestines and other sites. In vitro experiments also demonstrated that bilirubin impairs the normal coagulation mechanism and causes morphological changes and aggregation of platelets (Suvansri, 1969; Maurer and Caul, 1972). Alteration in platelet function may therefore be a key mechanism in the haemorrhagic and thrombotic disorders caused by increased bilirubin concentrations. However, the effect of bilirubin on different platelet parameters has not yet been analysed in detail.

One important parameter is the electric charge on the platelet surface. It is generally accepted that the surface charge of platelets, which can be measured by determining their electrophoretic mobility (EM), plays an important role in platelet aggregation (Seaman and Vassar, 1966; Marmur et al., 1976). Since platelet aggregation may be considered a trigger mechanism for thrombogenesis, analysis of the EM of platelets under various pathological conditions seemed a promising approach for further clarification of the thrombotic or haemorrhagic complications seen in sick babies. We decided therefore to study the action of bilirubin on the electrophoretic behaviour of platelets from human newborn under various conditions in vitro.

Material and Methods

Cord blood was drawn from the placental end of the cord after normal delivery at term and mixed 9:1 with sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 300 g for 15 min at room temperature. Washed platelets in suspension (WP) were obtained as published elsewhere (Kosztolányi et al., 1977); washing was carried out in Tris-buffered saline, pH 7.8 (0.145 M NaCl adjusted to pH 7.8 with 0.145 M Tris-HCl). Plastic disposable test tubes and siliconized glassware were used. Experiments were accomplished within 6 h of blood collection.

Bilirubin stock solution was prepared by dissolving 15 mg crystallin bilirubin (Reanal, Hungary) in 1.5 ml 0.1 N NaOH and then adding 8.5 ml Tris-buffered saline (pH 7.8). This stock solution was made freshly before each test and kept in the dark.

Electrophoretic studies were performed either with WP or PRP. WP was prepared by resuspending the platelets (about 10^5/ml) after the final washing in 10 ml Tris-buffered saline, pH 7.8 containing 10^{-3} M $CaCl_2$. In some experiments $CaCl_2$ was replaced with 10^{-3} M $MgCl_2$. Various amounts of bilirubin stock solution were added to give the desired final bilirubin concentration and the samples were incubated for 10 min at room temperature. After 10 min the samples were introduced into an OPTON Cytopherometer and their EM was determined.

Studies on PRP were carried out as follows. Various amounts of bilirubin stock solution were added to 1 ml aliquots of PRP to give the desired final bilirubin concentrations. The samples were incubated for 10 min at room temperature and then, just before the electrophoretic measurements, were diluted 1:10 with Tris-buffered saline, pH 7.8 containing 10^{-3} M $CaCl_2$ to obtain a cell concentration of 10^5/mm^3.

The effect of bilirubin on the EM of WP was also tested in the presence of albumin. Human albumin (Calbiochem) was dissolved in Tris-buffered saline, pH 7.8 and various amounts of this 100 mg/ml stock solution were added to samples of WP prepared as described above. After 10 min incubation at room temperature, bilirubin was added to the samples in a final concentration of 7.5 mg/dl. The specimens were again incubated for 10 min at room temperature and then introduced into the cytopherometer.

Each test was performed in triplicate. All procedures were made in the dark.

EM of platelets was measured in an OPTON Cytopherometer at 4 mA current intensity and at 25°C. After measurements, the specific resistance of each suspending medium was determined. In each sample the migration of at least 20 cells was measured in both directions.

The aggregation of platelets were estimated under the microscope. *Microscopic aggregation* means that clumps (about 2—10 platelets) could only be seen under a microscope; *macroscopic aggregation* means that clumps could be observed by simple inspection of the suspension.

Results

The effect of bilirubin on the EM of WP is illustrated in Figure 1. Bilirubin markedly increased the negativity of the platelets, even at a concentration as low as 1.5 mg/dl. A tendency for platelets to aggregate was also observed and this was so extensive that the EM of platelet clumps could not be determined at a bilirubin level higher than 7.5 mg/dl. On washing the platelets three times in Tris-buffered saline, pH 7.8, the EM was restored to the initial value. These bilirubin effects showed an absolute requirement for calcium ions and no change in the EM or aggregation could be observed when calcium ions were replaced by magnesium.

Figure 2 shows the effect of bilirubin on the EM of platelets in the presence of plasma. Plasma components inhibited the action of bilirubin up to 19.5 mg/dl, and only a small increase in negativity—as well as weak aggregation—occurred at bilirubin levels of 22.5 mg/dl or higher.

Figure 3 and Table 1 illustrate the effect of albumin on the bilirubin-induced change in the mobility of WP. Normally, the serum bilirubin binding capacity of newborns is saturated at bilirubin levels of 22—23 mg/dl, i.e. at a bilirubin/albumin molar ratio of about 0.8 (Cashore et al., 1978) which implies that 1000 mg albumin can bind 8 mg of bilirubin. We found that at a bilirubin/albumin molar ratio of 0.88, the action of bilirubin on platelet EM was abolished. When the saturation of albumin with bilirubin was exceeded, and presumably

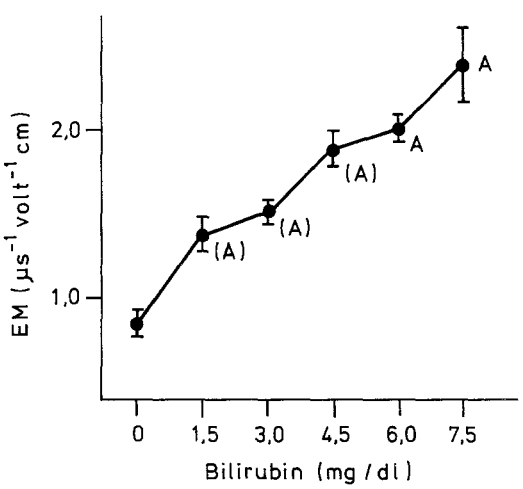

Fig. 1. Effect of bilirubin on the electrophoretic mobility (*EM*) (mean ± SD) of washed platelets. A: macroscopic aggregation; (*A*): microscopic aggregation

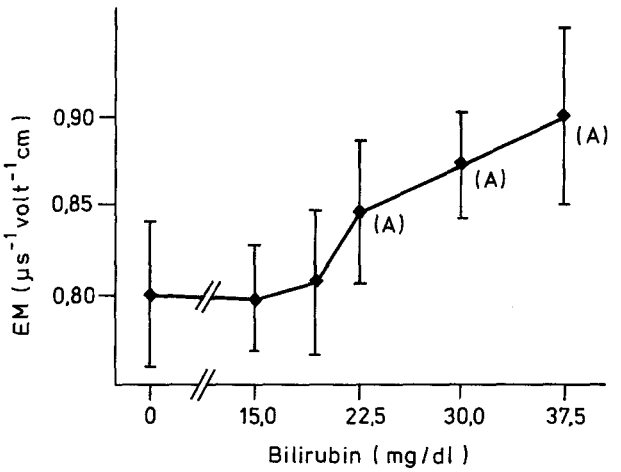

Fig. 2. Effect of bilirubin on the electrophoretic mobility (*EM*) (mean ±SD) of platelets suspended in dilute plasma. (*A*): microscopic aggregation

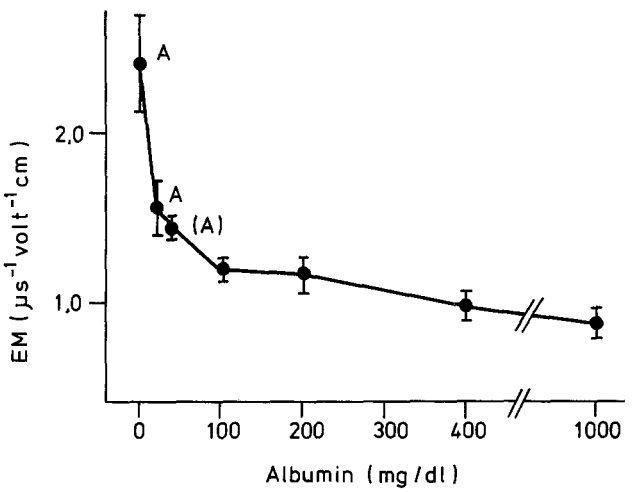

Fig. 3. The albumin dependence of the increase in electrophoretic mobility (*EM*) (mean ±SD) of washed platelets exposed to 7.5 mg/dl bilirubin. *A*: macroscopic aggregation; (*A*): microscopic aggregation

free bilirubin was present (bilirubin/albumin molar ratios 2.2 or higher), platelet negativity increased. The slope of the curve in Figure 3, however, shows that the fraction of bilirubin which could be expected to be free over the saturation point does not have the same effect as free bilirubin in the complete absence of albumin. For example, where albumin at a concentration of 100 mg/dl was presumably able to bind only 0.8 mg bilirubin out of the 7.5 mg per dl, the free bilirubin fraction (calculated to be 6.7 mg per dl) failed to have the same effect as that concentration of truly free bilirubin in the complete absence of albumin (Fig. 1).

Table 1. The albumin dependence of the increase in electrophoretic mobility (EM) (mean ±SD) of washed platelets exposed to 7.5 mg/dl bilirubin

Albumin mg/dl	Bilirubin mg/dl	EM $\mu sec^{-1} volt^{-1} cm$	Bilirubin/albumin molar ratio
0	7.5	2.41 ± 0.28	—
20	7.5	1.55 ± 0.16	44.13
40	7.5	1.44 ± 0.06	22.07
100	7.5	1.19 ± 0.10	8.82
200	7.5	1.17 ± 0.06	4.41
400	7.5	0.97 ± 0.10	2.20
1000	7.5	0.87 ± 0.08	0.88
0	0	0.86 ± 0.08	—

Discussion

In the experiments reported here, bilirubin markedly increased the negative EM of WP. The increase in negativity was calcium dependent and could be abolished by washing the platelets, indicating that bilirubin did not cause irreversible damage to the platelet surface. The results suggest that the negative bilirubin molecules became attached to the platelet surface, probably through calcium ions.

We observed a concentration-dependent aggregation of WP, in parallel to the increase in negativity. This observation was surprising, since it is generally believed that the surface negativity is somehow reduced when platelets aggregate (Seaman and Vassar, 1966; Marmur et al., 1976). Maurer and Caul (1972) have also observed platelet aggregation following incubation with bilirubin, and suggested that ADP released from the platelets is probably the cause. However, they did not determine the EM of platelets exposed to bilirubin, and their suggestion appears unlikely because ADP molecules decrease the surface negativity when adsorbed (Seaman and Vassar, 1966), in contrast to our findings. We can explain the phenomenon on the assumption that clumping takes place between platelets carrying bilirubin molecules on their surface. The bilirubin-induced aggregation, being calcium dependent, might be attributed to bilirubin-calcium bridges joining adjacent platelets in the clump. The fact that calcium ions could not be replaced by magnesium ions indicates the specificity for calcium in this phenomenon.

When tests were performed on platelets suspended in plasma, bilirubin action could first be detected at concentration of 22.5 mg/dl, indicating that bilirubin could not attach to the platelet surface below this level. This concentration approximately coincides with that of bilirubin at the saturation point of the serum bilirubin-binding capacity in normal infants, as determined with other methods. Thus, electrophoretic investigations on platelets could provide information about the plasma bilirubin-binding capacity and indicate the appearance of free bilirubin in the system.

Surprisingly, bilirubin concentrations above the saturation point caused only a small increase in the platelet negativity and in the aggregation tendency (Fig. 2),

whereas a marked increase in both parameters, as shown in Figure 1, might have been expected. The results of experiments with albumin (Fig. 3) also indicate that the fraction of free bilirubin exceeding the albumin saturation point did not have the same effect as that concentration of truly free bilirubin in the complete absence of albumin.

Electrophoretic measurements demonstrated a marked difference between washed platelets and platelets in the presence of plasma (Turpie et al., 1972). Plasma constituents, if adsorbed, could influence the EM (Seaman, 1975). It may be that protein molecules are taken up by platelets suspended in a protein-containing medium such as plasma. We think that albumin molecules loosly adsorbed onto the surface may protect the platelets against the attachment of free bilirubin, even when the bilirubin concentration exceeds the serum bilirubin-binding capacity.

In a recent in vitro study we have observed a significant decrease in platelet EM induced by acidotic pH values which are frequently seen in sick neonates (Kosztolányi and Jobst, 1978). Since the change in surface charge was found to be plasma dependent, we suggested that it might be the result of an abnormality in the platelet-plasma protein interaction caused by acidosis. It is not unrealistic to assume that the protective effect of albumin against bilirubin might also be impaired in acidosis. If this is really the case, the above effects of bilirubin on platelets could contribute to the haemostatic abnormalities in sick babies with both hyperbilirubinaemia and acidosis.

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