Biochimica et Biophysica Acta, 436 (1976) 366–376

BBA 77337

THE FUSION OF ABNORMAL PLASMA LIPOPROTEIN (LP-X) AND THE ERYTHROCYTE MEMBRANE IN PATIENTS WITH CHOLESTASIS STUDIED BY ELECTRONMICROSCOPY

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(Received December 23rd, 1975)

SUMMARY

Adhesion followed by fusion of LP-X vesicles with the erythrocyte membrane is an important contribution to the erythrocyte enlargement in patients with intra or extra hepatic cholestasis. Adhesion of LP-X vesicles is demonstrated by thin section and freeze-etch electronmicroscopy. Fusion of LP-X with the erythrocyte membrane is deduced from biochemical data and freeze-etch electronmicroscopy in that the uptake of cholesterol and lecithin coincides with the increase in smooth areas on the fracture faces of the erythrocyte membrane.

INTRODUCTION

In many hepatobiliary diseases an increase of the mean erythrocyte diameter is observed and is accompanied by an increased resistance of the red cells to osmotic lysis (for a review see ref. 1). Chemical analyses have shown that the increase in cell diameter and surface area is associated with an increased content of cholesterol [2–4] and of phospholipid [4–7] in the red cell membrane. Transfusion experiments have shown that both the morphological and chemical changes occur in the blood circulation and are reversible [5, 8].

More recent investigations have revealed that patients with intra or extra hepatic cholestasis have an abnormal plasma lipoprotein which is commonly called LP-X [9, 10]. This LP-X contains predominantly cholesterol and phosphatidylcholine and only 6 % protein [9–13]. Electronmicroscopy shows LP-X as small vesicles bounded by a lipid bilayer [13]. It has been suggested that LP-X plays a role in the acquiring of an increased lipid content of circulating erythrocytes in patients with cholestasis [14].

In this paper we report on the results of a freeze-etch electronmicroscopic study of the red cell membranes from patients with cholestasis as compared to normals. The freeze-etch technique detects changes in the lipid/intrinsic protein ratio,

as reflected by the particle density on the fracture faces of the membrane [15]. The correlation of the electronmicroscopy and the chemical analysis of pathological red cell membranes and LP-X have led to a hypothesis concerning the molecular mechanism responsible for the enlargement of the erythrocytes in these patients.

METHODS

Fresh heparinised samples of erythrocytes taken from patients with intra or extra hepatic cholestasis and controls (after an overnight's fast) were centrifuged for 10 min at $2000 \times g$. Lipids were extracted according to the method of Reed et al. [16]. Cholesterol and phospholipid analysis of the extracts were carried out according to the method of Huang et al. [17] and a modified procedure of Fiske and SubbaRow [18] respectively. The phospholipids were separated by thin-layer chromatography and phosphorus analysis was carried out on the separated spots. For freeze-etch electronmicroscopy the cells were quenched immediately after centrifugation in a mixture of liquid and solid nitrogen and prepared further in a Denton freeze-etch apparatus as described before [19]. For thin sectioning (positive staining), the cells were fixed with the tricomplex fixation followed by OsO_4 fixation [20]. Micrographs were made with a Siemens Elmiskop IA and a Philips 301.

The following hematological values were measured and calculated (cf. ref. 21).

Mean cell diameter (MCD) was determined by the method described by Haden [22]. The values found in normal subjects were between 7.4 and 7.9 μm.

Mean cell thickness (MCT) was calculated according to the equation $MCT = 4 \times MCV/\pi \times (MCD)^2$ μm. The values found in the normal subjects were between 1.9 and 2.4 μm (MCD and MCV expressed in μm and femtolitres (fl, $1 \cdot 10^{-5}$ l).

Mean cell volume (MCV) values found in the normal subjects were between 85 and 104 fl.

Mean cell surface area was evaluated according to the formula Mean Cell Surface area $= 0.5\,\pi(MCD^2) + \pi \times (MCD) \times (MCT)$. The values found in normal subjects were between 139 and 150 μm^2.

The abnormal lipoprotein (LP-X) in the serum was detected by electrophoresis in agar and precipitated with poly-anions [23]. The LP-X was also visualized by negative staining electronmicroscopy using ammonium molybdate 1 % pH 6.5. For an in vitro incubation experiment LP-X was isolated and purified according to Seidel [10].

RESULTS

Previous work has shown [2–7] that the cholesterol and phospholipid content of the erythrocyte membrane increases in patients with intra and extra hepatic cholestasis. This is confirmed by new data given in Fig. 1, which show that the total surface area per cell enlarges coincident with increases in cholesterol and phospholipid.

To explore the consequences of the lipid increase on the membrane morphology we compared freeze fracture faces of normal cells with cells from severe cases of cholestasis in which the mean surface was increased to 170 μm^2. Fig. 2A shows the inner fracture faces of a normal erythrocyte and Fig. 2B, C and D inner fracture faces of pathologically enlarged cells. The following changes were routinely observed in abnormal cells:

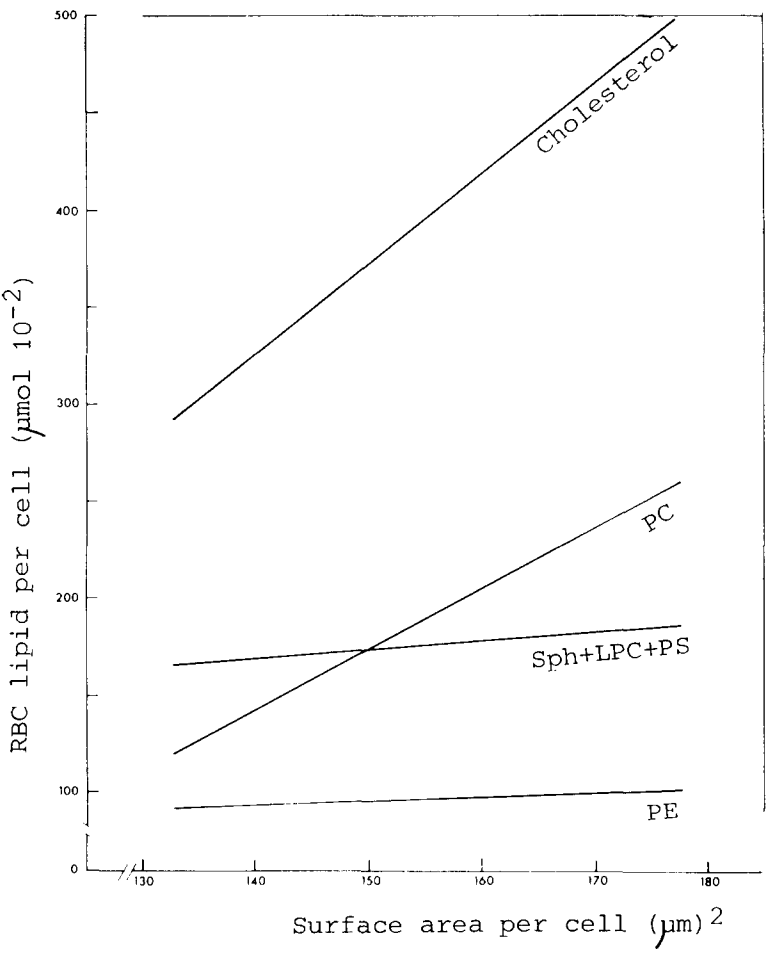

Fig. 1. The lipid content per erythrocyte in relation to the surface area per erythrocyte in patients with intra or extra hepatic cholestasis. Note the almost similar slope of cholesterol and lecithin. Increase of cholesterol and lecithin is statistically highly significant.

(i) The intramembraneous particles are not homogeneously distributed on the fracture faces. This is especially clear on the inner fracture faces. There are various gradations of particle aggregation present in the same population of enlarged erythrocytes.

(ii) The particle density found on the inner fracture faces of the pathological erythrocytes is significantly lower. It is apparent that the particle density on the fractured area in Fig. 2D is lower than in Fig. 2A. Pathological cells from a population having a mean surface area of 170 μm^2 had a particle density count of $2350 \pm 300/\mu m^2$ as compared to normal values of $3000 \pm 200/\mu m^2$ (this last value is in good agreement with earlier data [24]). From these values a particle density dilution factor of 1.25 was calculated.

Furthermore the freeze-etch morphology at different stages of the disease has been investigated. The morphological characteristics of the early stage of cell en-

largement are very striking. At this stage the cell-diameter (surface area/cell) is still normal and LP-X is present in the serum. Fig. 3 shows a typical micrograph for the early stage. The particle distribution on the fracture faces is normal, but smooth areas (400–800 Å in diameter) are frequently present on the fracture faces. Prints of these structures can also be observed on the etch faces and occasionally such a struc-

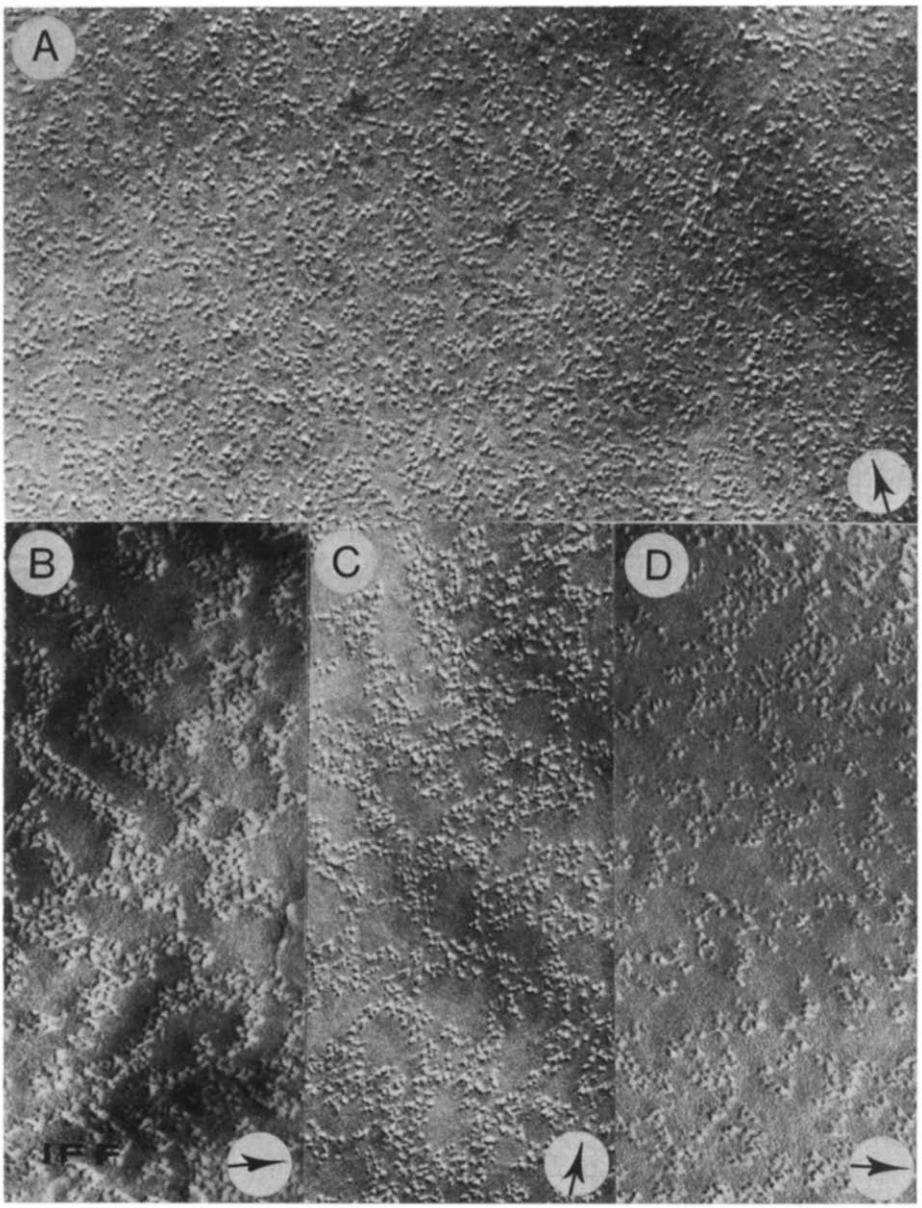

Fig. 2. Inner fracture faces of a normal erythrocyte membrane (A) and of erythrocyte membranes out of a population of pathological cells (B, C and D) (×90000).

ture is visible on the edge of an etch and a fracture face (arrow Fig. 3A). Furthermore, it has to be noted that on the inner fracture faces the observed elevations are associated with complementary impressions on the outer fracture faces.

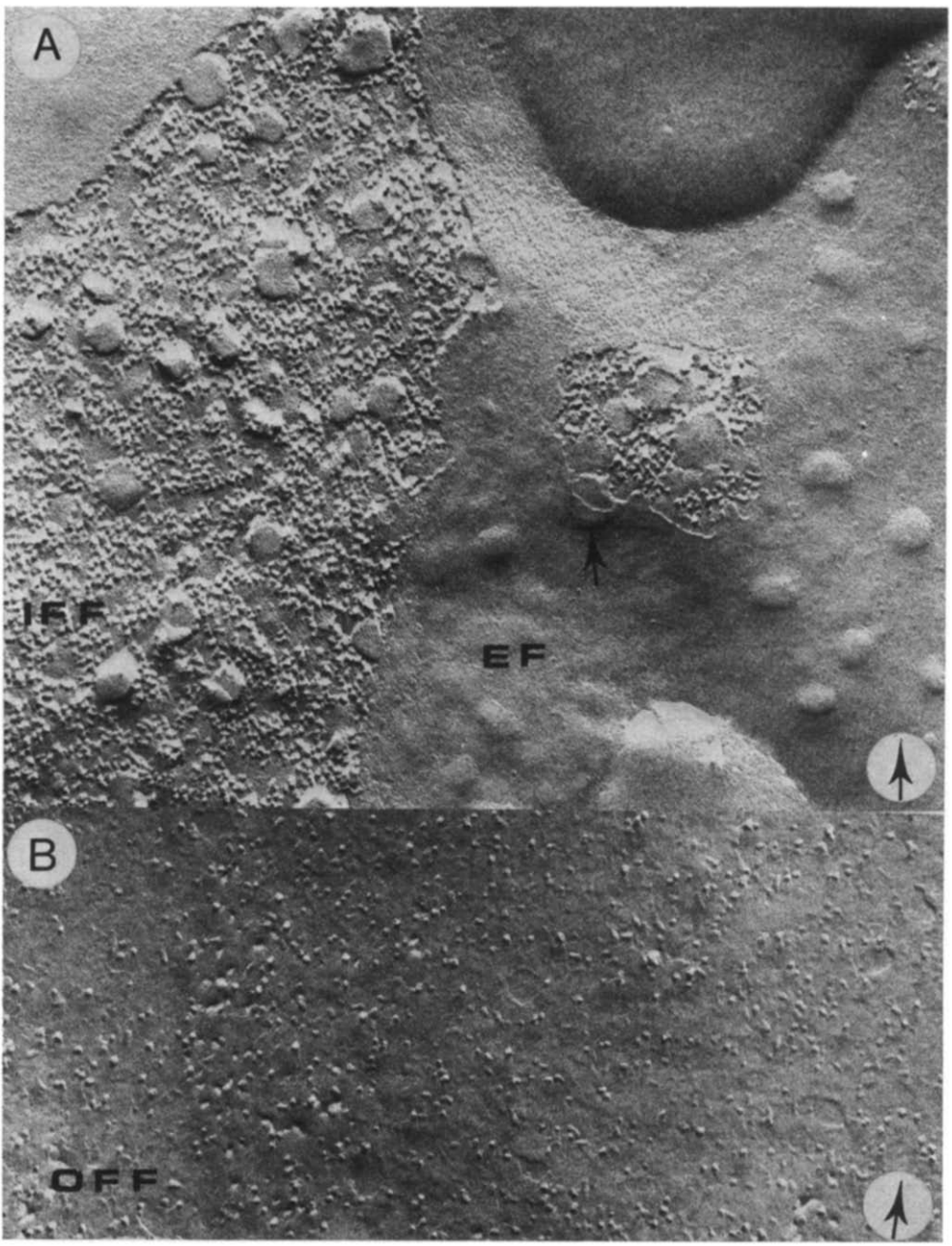

Fig. 3. Inner (A) and outer (B) fracture face of the initial stage of the enlargement of pathological cells (×100000).

In addition to this in vivo investigation we have conducted experiments to elucidate whether similar alterations can be induced in vitro and whether the abnormal lipoprotein LP-X is involved in the enlargements of the erythrocytes. Therefore we have incubated normal erythrocytes with compatible serum of a patient in which LP-X was present. The diameter of the erythrocytes increased from 7.4 ± 0.2 to about 8.2 ± 0.2 ($n = 10$) and the cholesterol and phospholipid content per erythrocyte was significantly increased.

Freeze etching shows that the fracture faces of the erythrocytes after 2 min of incubation are characterized by smooth structures (400–800 Å in diameter) as described for the early stage of the erythrocyte enlargement noted in vivo (Fig. 3). The particle distribution is still normal. After 2 h one observes particle aggregation and a significant decrease in particle density on the inner fracture faces from $3000 \pm 200/\mu m^2$ to $2500 \pm 300/\mu m^2$ (Fig. 4).

Moreover, we have incubated LP-X, purified according to the method of Seidel [10] (see negative staining of the LP-X fragment in Fig. 5C), with normal erythrocytes. No increase in cell diameter could be found. Freeze etching shows clearly that similar smooth structures (400–800 Å in diameter) are present on the fracture faces. These smooth areas are elevated exclusively on the inner fracture faces

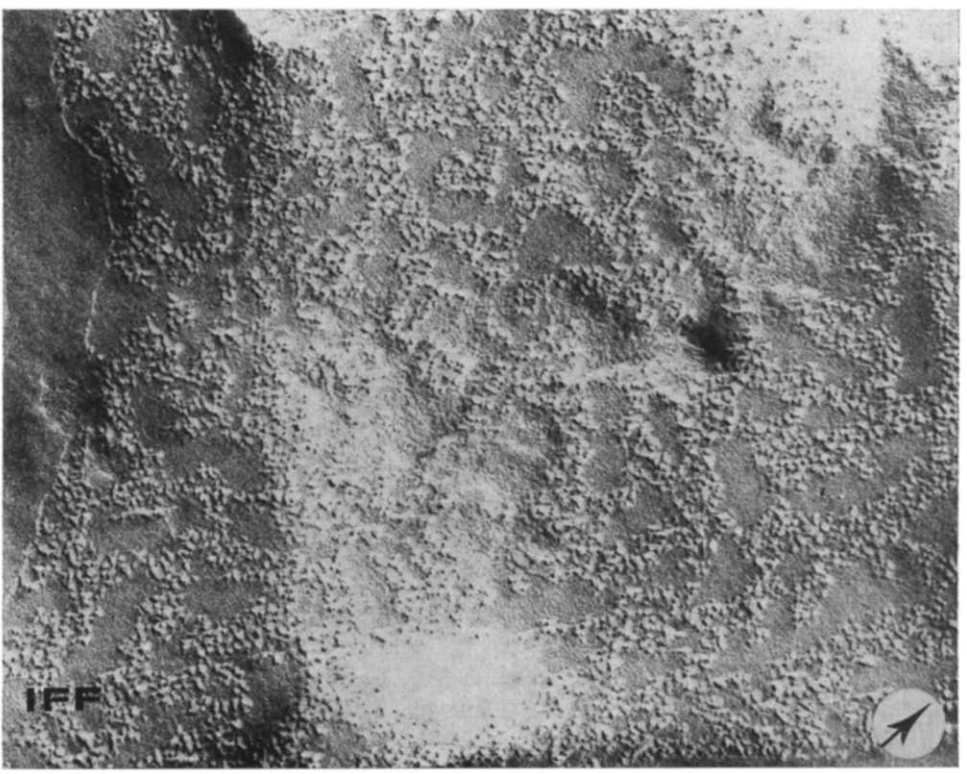

Fig. 4. Inner fracture face of an erythrocyte incubated in vitro during 2 h in compatible LP-X serum (×100000).

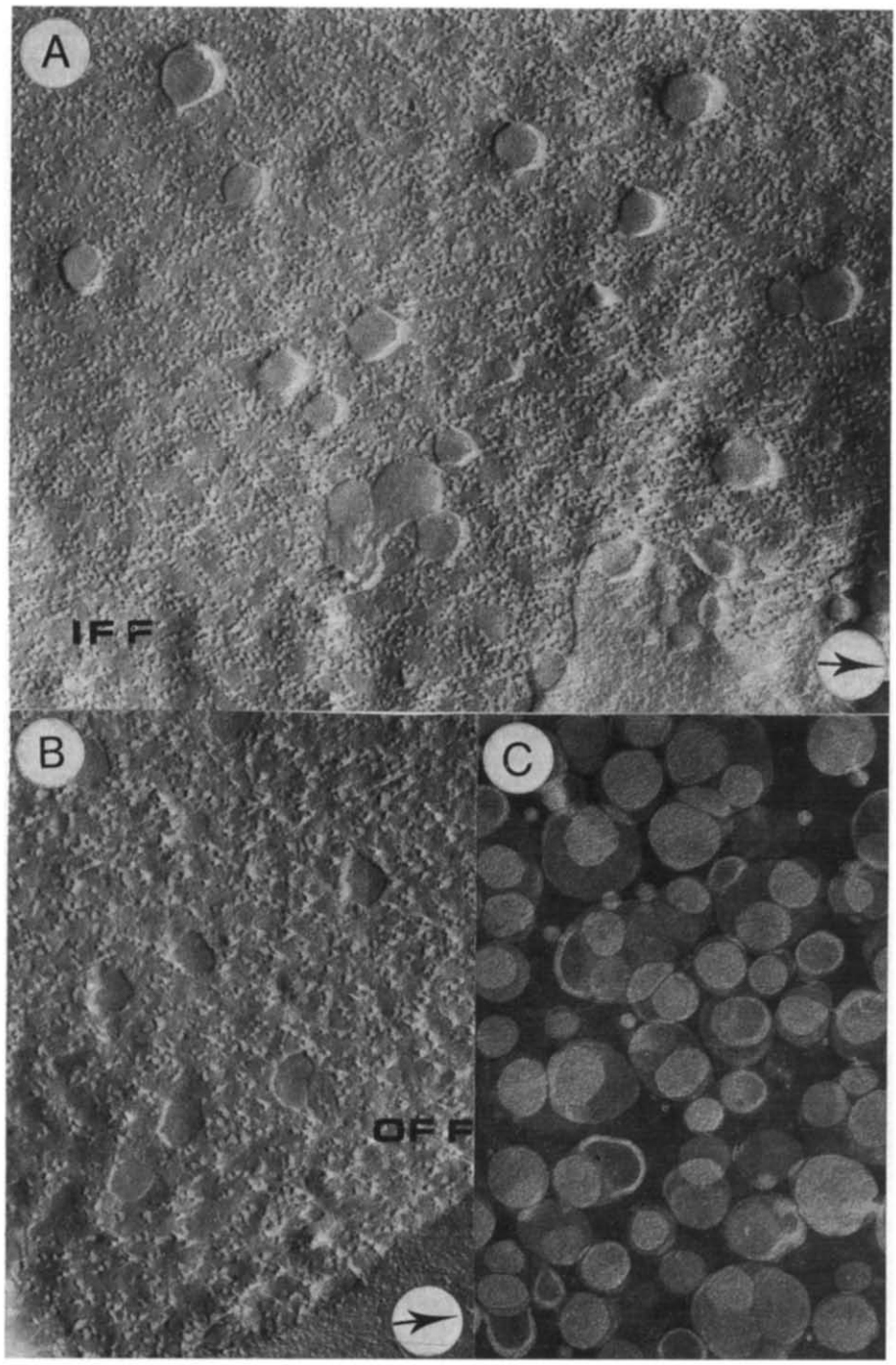

Fig. 5. Inner (A) and outer (B) fracture face of erythrocytes incubated in vitro during 2 h in LP-X, purified by the method of Seidel [10]. A negative staining preparation of the pure LP-X (C) ($\times 100000$).

with complementary impressions present on the outer fracture faces. The particle distribution is unaltered (Fig. 5A and B).

As a consequence of these results we made thin sections of material from both the in vivo and in vitro experiments. The two characteristic freeze-etch stages were investigated.

(1) The initial stage; normal erythrocyte size and particle distribution on the fracture faces, but the characteristic smooth structures (400–800 Å in diameter).

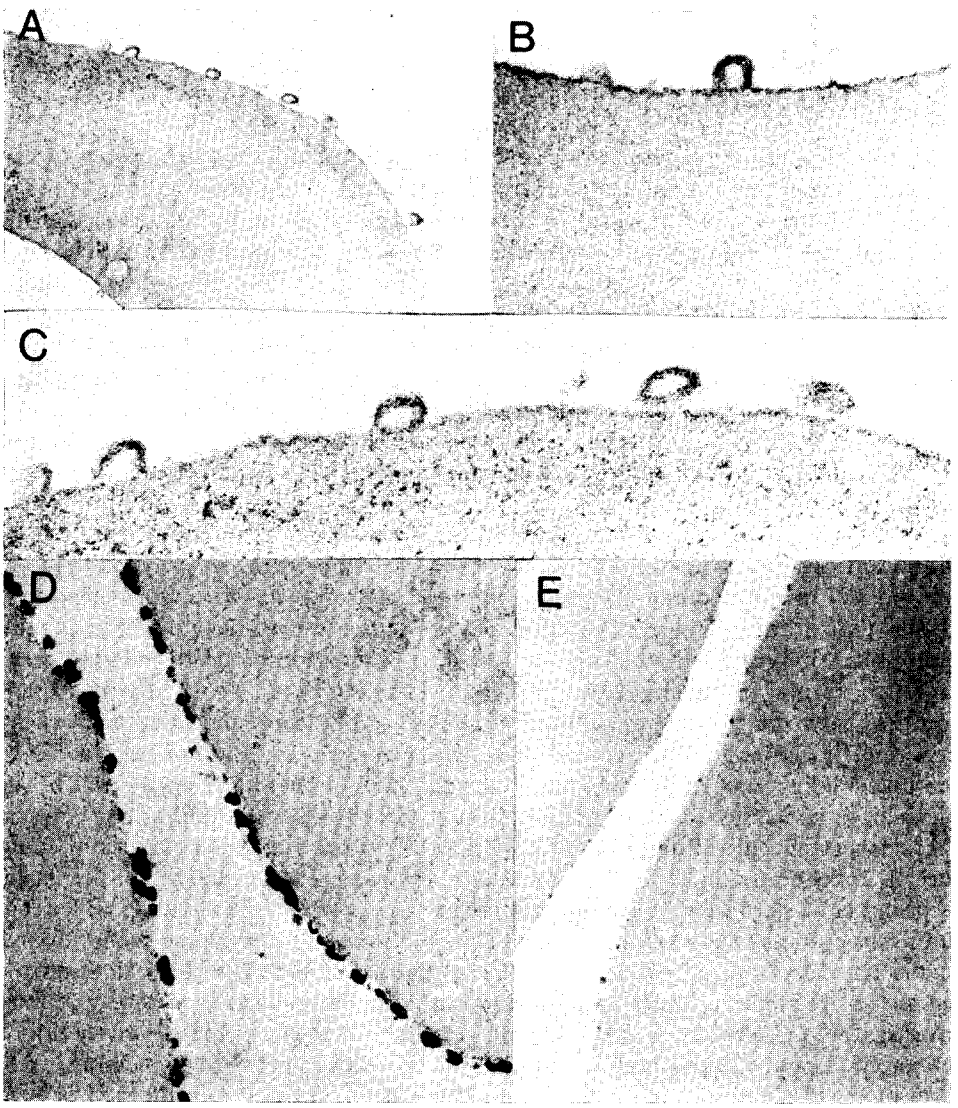

Fig. 6. Thin sections of erythrocytes in the initial stage of the enlargement (A, B and C in vivo and D in vitro) and of erythrocytes at the end stage of the enlargement (E). (A, D and E × 27000, B and D × 90000).

(2) The end stage; erythrocyte enlargement and the dilution of the particles on the fracture faces.

Since fixation with glutaraldehyde and OsO_4 did not reveal the LP-X structures, the tricomplex fixation was used. Before fixation the cells were washed with 0.9 % NaCl.

Fig. 6B and C show an erythrocyte from a patient at the initial stage of the enlargement. The mean diameter of the cells was 7.9 μm. Fig. 6D shows erythrocytes incubated for 2 min in LP-X serum, Fig. 6E shows the end stage of both the in vivo and in vitro situation. The vesicles found at the initial stage of the enlargement are about 500 Å in diameter. These vesicles are rarely found at the end stage of the enlargement (Fig. 6E).

DISCUSSION

The increase in surface area of erythrocytes from patients with cholestasis can be explained by the insertion of cholesterol and lecithin into the erythrocyte membrane. This increase in surface per cell explains the increase in cell diameter and the increased osmotic resistance as the cell can swell to a larger critical sphere in hypotonic solutions. This view is supported by the results of the freeze-etch electronmicroscopy of erythrocyte membranes from cholestasis. The mean particle density reflecting the number of intrinsic protein complexes, is reduced whereas the total membrane area is increased. Furthermore it was found that the content of sialic acid per erythrocyte is not altered during the enlargement of the cell (personal communication, R. F. A. Zwaal). These observations permit us to conclude that the increase in surface area is due to the insertion of lipids, whereas the total amount of intrinsic membrane protein per cell is similar.

The mechanism by which the increased lipid content is acquired, is also an important concern. It is well known that direct relations exist between the lipids of the red cell membrane and the plasma lipoproteins. Transfusion experiments have shown that normal circulating erythrocytes of patients with liver diseases acquire increased osmotic resistance within a few days whereas osmotically resistant cells revert to normal in the circulation of healthy subjects [5, 8]. From in vitro experiments it is known that there is a rapid exchange of free cholesterol and lysolecithin between the plasma and the red cells [14]. Moreover, the acylation of lysolecithin to the diacyl compound in the membrane and the flow of lecithin from the cell to the plasma are processes which contribute to a complex exchange equilibrium between the plasma and red cell lipids (cf. ref. 14). A disturbance of this equilibrium could directly influence the lipid levels in the red cell. A change in the activity of the serum lecithin : cholesterol acyltransferase will effect the levels of free cholesterol, lecithin and lysolecithin in the plasma and consequently through the exchange processes also in the red cell. Cooper [6] has pointed out that in patients with liver diseases the activity of lecithin : cholesterol acyltransferase can be decreased. However, other investigators [23] showed that the lecithin : cholesterol acyltransferase activity can be enhanced. Thereby, it has been shown that the correlation between the lecithin : cholesterol acyltransferase activity and the lipid abnormalities in the erythrocyte is poor [5, 8]. So it can be assumed that other important factors are involved as well.

It has been proposed that the abnormal lipoprotein LP-X is responsible for the increase of cholesterol and lecithin in the plasma of the patients with cholestasis [10].

It is plausible that this lipoprotein is involved in the interplay between plasma and cells. As outlined in the introduction LP-X consists of small (300–600 Å in diameter)

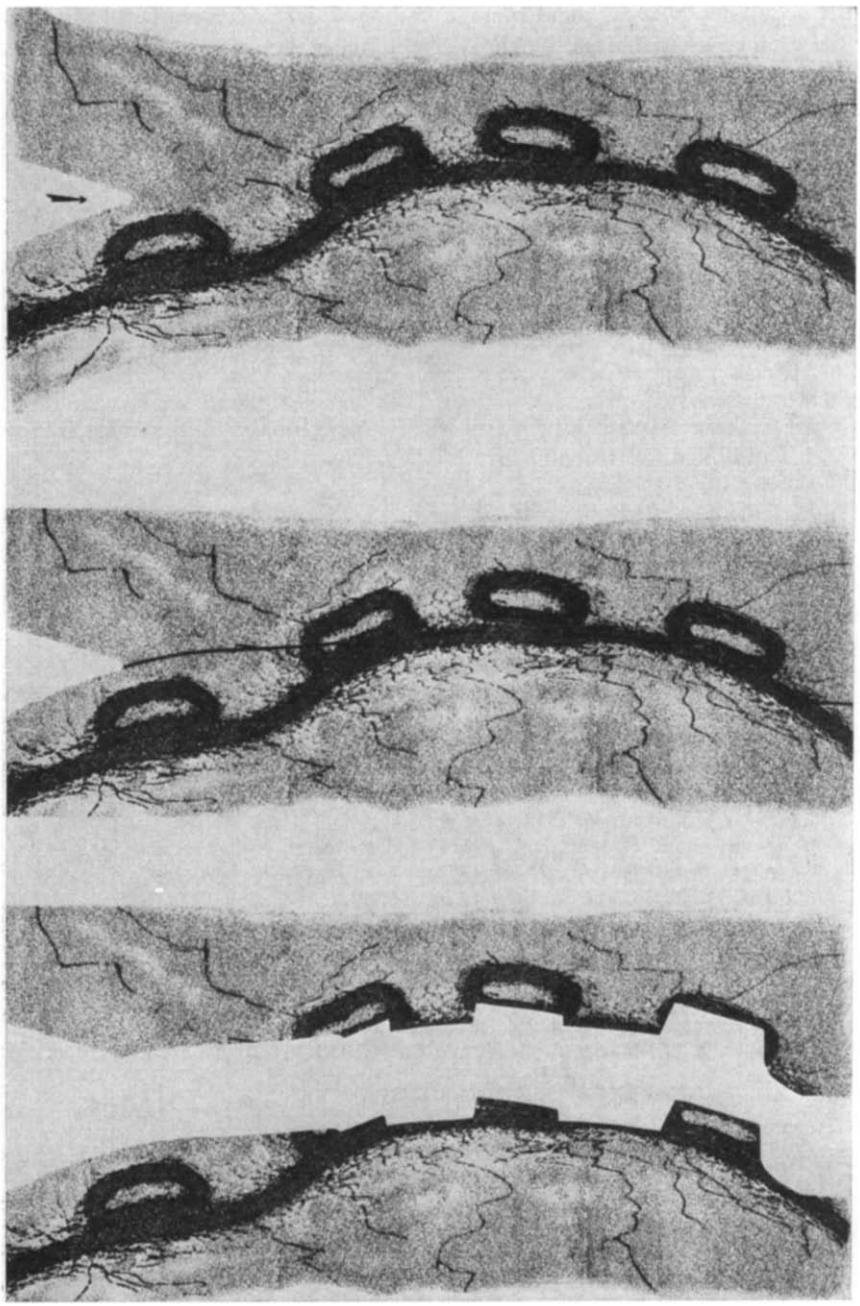

Fig. 7. Schematic drawing of the fracture plane through the erythrocyte membrane at the initial stage of the enlargement.

single bilayer vesicles composed primarily of cholesterol and lecithin molecules in a 1 : 1 ratio [13]. The small amount of protein gives the vesicles a positive charge as determined by their electrophoretic behaviour [23]. This characteristic promotes adhesion to the negatively charged membrane, a finding which is evident from the thin section of the washed erythrocytes. Analysis of the freeze-etch micrographs strongly supports the conclusion that adhesion precedes the enlargement process both in vivo and in vitro with LP-X serum. The adhered LP-X vesicles appear as smooth areas (400–800 Å in diameter) on the fracture faces and correspond to fracture faces through the bilayer of the vesicles. The fracturing model elucidates why smooth areas on the fracture faces and the complementary impressions on the outer fracture faces are present (Fig. 7). The observed dilution of the intramembraneous particles adds further support to the view that fusion of the LP-X vesicles with the red cell membrane contributes significantly to the increase in erythrocyte surface area of patients with cholestasis.

ACKNOWLEDGEMENTS

We want to thank Mrs. J. Bijvelt and Mr. F. Neys for the technical assistance and Mr. N. M. van Galen for drawing Fig. 7.

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