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ERYTHROCYTE MEMBRANE CHOLESTEROL LEVELS AND THEIR EFFECTS ON MEMBRANE PROTEINS

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Summary

The susceptibility of the band 3 protein of the erythrocyte membrane to proteolytic digestion at either surface of the membrane was not altered when the membrane cholesterol level was increased by 65—103%. Cross-linking of the major membrane proteins by *o*-phenanthroline · Cu, glutaraldehyde, dimethylsuberimidate and dimethyladipimidate was also unaffected.

The cholesterol content of the red blood cell membrane can be greatly increased in certain diseases or after cholesterol feeding [1, 2]. Such changes can also be achieved by suitable treatment of the cells in vitro [3]. It has been proposed [4] that the position of integral membrane proteins in the lipid bilayer is altered when the level of cholesterol in the membrane is increased or decreased. We have used trypsin and chymotrypsin to see whether, following cholesterol enrichment, any difference in the degree of exposure of the band 3 protein at either membrane surface could be detected.

It has been calculated that a doubling of the erythrocyte membrane cholesterol content increases its area by 20—25% and increases of this order result in a gross distortion of the cell shape [3]. We have therefore also used protein cross-linking reagents to investigate whether any changes in the relationships of the major proteins associated with the membrane could be detected.

Human red blood cell cholesterol levels were increased by incubation with cholesterol-rich plasma [5] while control cells were incubated with nor-

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Abbreviation: SDS, sodium dodecyl sulphate.

mal plasma under the same conditions. Whole cells were digested with chymotrypsin, and their ghosts [6], with trypsin [7], using phenylmethylsulphonyl fluoride (1 mM) to terminate the reaction. Ghost membrane proteins were cross-linked with *o*-phenanthroline · Cu chelate [8], dimethyladipimidate and dimethylsuberimidate under the conditions described by Ji [9] and with glutaraldehyde. The latter was used at both 0.015% [10] and 1% [11] concentrations. The proteins were separated by SDS-polyacrylamide gel electrophoresis [12] and the gels were scanned at 554 nm in a Gilford 240 spectrophotometer fitted with a linear transport attachment. Other methods were as previously described [13].

Table I shows the results of enriching the erythrocytes and ghosts with cholesterol. The cell preparations for treatment with chymotrypsin showed a 65—77% increase in cholesterol content while the ghost membrane preparations for treatment with trypsin showed a 93—103% increase.

TABLE I

ENRICHMENT OF ERYTHROCYTES AND THEIR GHOSTS WITH CHOLESTEROL

Preparation	Supsequent treatment	Cholesterol/phospholipid molar ratio	
		Control cells	Enriched cells
Whole cells	digestion with chymotrypsin	1.00 1.03	1.77 1.70
Cell ghosts	digestion with trypsin	1.07 0.97	2.06 1.97

The effects of digestion with the two proteases are shown in Fig. 1. As others have reported [7], treatment of intact erythrocytes with chymotrypsin results in the breakdown of the band 3 protein to give a major fragment with molecular weight of approx. 55 000 as judged from its position in the gel. Recovery of another less prominent fragment, mol.wt. approx. 38 000, is reported to be low and very variable [6] and we did not detect it in our experiments. In both experiments, the major band 3 fragment remaining after digestion of the cholesterol-enriched cells ran in the same position on the gels as that produced after digestion of the control cells.

Trypsin does not attack the band 3 protein in intact red blood cells but it attacks the protein in unsealed ghosts leaving behind a fragment, the apparent molecular weight of which is approx. 52 000 [7]. This presumably contains regions of the protein exposed at the outer surface and buried within the lipid bilayer. Fig. 1 shows that, with both control and cholesterol-enriched membranes, trypsin digestion results in the disappearance of most of the band 3 protein and the appearance of a major new band corresponding to the remnant reported by Steck et al. [7]. The extrinsic proteins on the inner surface of the membranes are also degraded, the most noticeable effect being the loss of much of the spectrin bands.

We have also studied the cross-linking of erythrocyte membrane proteins by various reagents. No difference could be detected in the peptide patterns obtained on gel electrophoresis of cross-linked membranes from control cells and cells in which the cholesterol content had been increased by 70—100%. Thus, after *o*-phenanthroline · Cu chelate treatment, band 3 molecules are largely

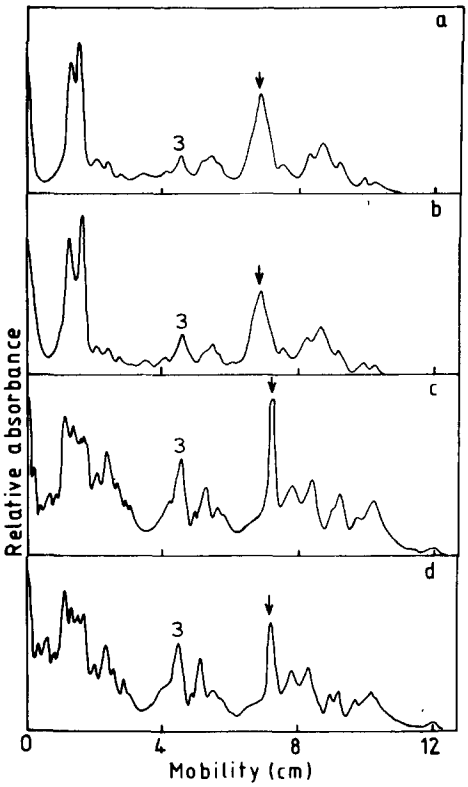

Fig. 1. Electrophoretograms of erythrocyte membrane proteins after proteolytic digestion. After incubation of whole cells with chymotrypsin, inhibitor was added and the cells were washed three times with phosphate-buffered saline before the ghost membranes were prepared [6] for electrophoresis. (a) Control cells incubated with chymotrypsin. (b) Cholesterol-enriched cells incubated with chymotrypsin. (c) Control ghosts incubated with trypsin. (d) Cholesterol-enriched ghosts incubated with trypsin. The position of the intact band 3 protein is indicated by the figure 3. The arrows indicate the position of the major residue from band 3. Each experiment was performed twice with very similar results.

cross-linked into dimers and the spectrin bands are much reduced in intensity [8, 14]. After mild glutaraldehyde fixation, bands 1, 2, 4.1, 4.2 and 7 were reduced in intensity with the appearance of high molecular weight material at the top of the gel; after more rigorous fixation (1% glutaraldehyde) no protein entered the gel. The main effects of cross-linking with the bis-imidate reagents were loss of much of the spectrin I and II bands with the appearance of high molecular weight material at the top of the gel [8, 15]. Smaller changes occurred in other bands but there was no obvious difference between the control and cholesterol-enriched membranes.

The fact that no difference could be detected in the accessibility to proteases of those regions of the band 3 protein exposed at either the cytoplasmic or the outer surface of the membrane suggests that any movement of the molecule into or out of the bilayer, following cholesterol enrichment, must be very small. It has been reported that exposure of erythrocyte membrane proteins to small fluorescence quenching agents or to non-penetrating reagents reacting with sulphydryl groups, is appreciably altered following an increase or decrease in the membrane cholesterol [16, 17]. It is not known

how many times the band 3 peptide chain crosses the membrane [18], but it might be expected that any major change in the position of the protein with respect to the bilayer would increase or decrease the number of sites of proteolytic attack at one surface or the other. As there was no such change, it seems probable that the marked changes in accessibility to the smaller reagents are the result of a conformational change rather than a movement of the whole molecule into or out of the bilayer.

The results obtained with cross-linking reagents complement our earlier finding [5] that uptake of cholesterol by the red blood cell was not affected by glutaraldehyde fixation. The nature of the attachment of spectrin and actin to the membrane has not been fully established. Although the basic attachment appears to be a protein-protein link [19], spectrin does interact with phospholipids, especially those present at the inner surface of the erythrocyte membrane bilayer, and appears to restrict their lateral and transmembrane diffusion [20, 21]. However, such protein-lipid interactions do not appear to restrict the ability of the bilayer to expand by up to 20% and no change in protein-protein interactions was detected following such expansion. Thus, the lipid bilayer and the protein framework of the membrane appear to have a considerable degree of independence from each other.

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