Biochimica et Biophysica Acta, 945 (1988) 105–110
Elsevier

BBA 70581

BBA Report

The distribution and aggregatability of intramembrane particles in phenylhydrazine-treated human erythrocytes

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(Received 16 June 1988)

Key words: Intramembrane particle; Freeze-fracture; Phenylhydrazine; Fluorescence, oxidant-induced; (Human erythrocyte)

Freeze-fracture analysis of phenylhydrazine-treated, unfixed human erythrocytes showed a random distribution of intramembrane particles both over membrane-bound Heinz-bodies and in the intervening areas when examined after fast freezing in liquid propane. The same results was obtained when unfixed, glycerinated red cells were frozen in liquid Freon. In contrast to previously published data (Low et al. (1985) Science 227, 531–533) these results indicate that binding of Heinz-bodies to the red cell membrane cannot cause morphologically detectable clustering of Band 3 in phenylhydrazine-treated red cells. Over numerous Heinz-bodies a decreased Acridine orange-induced particle aggregation was observed. The phenomenon of the oxidant-induced red cell fluorescence is described.

In recent years haemoglobin–erythrocyte membrane interaction has been the subject of considerable investigation. The cytoplasmic pole of the membrane-spanning protein Band 3 has been identified as the high-affinity binding site for haemoglobin [1–4]. More recently, interaction of denatured haemoglobin derivatives with the erythrocyte membrane was also investigated. Waugh and Low [5] reported that haemichromes induced by phenylhydrazine-treatment of haemoglobin have a much higher affinity for the cytoplasmic domain of Band 3 than native haemoglobin and that these haemichromes caused the isolated cytoplasmic segment of Band 3 to precipitate in solution. Phenylhydrazine treatment of intact erythrocytes induces substantially increased intracellular haemichrome formation both in vivo and in vitro [6]. This leads to the formation of Heinz-bodies which bind to the internal surface of the red cell membrane [7]. An increased quantity of membrane-bound haemoglobin was found in the red cells of patients with hereditary inclusion body anaemia where spontaneous haemichrome and Heinz-body formation occur [8]. In these diseases the increased quantity of membrane-bound haemoglobin was accompanied by an increase in the binding of autologous IgG to the red cell surface [8,9]. Using immunofluorescence microscopy Low et al. [10] suggested that Band 3 is clustered above the membrane-bound Heinz-bodies in phenylhydrazine-treated human erythrocytes. Furthermore, the autologous IgG bound to the outer red cell surface was also found to show colocalization with the Heinz-body-membrane attachment sites [10]. From these results the authors

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concluded that the Heinz-body-induced clusters of Band 3 molecules constitute the binding site for the autologous IgG. The increased binding of IgG might result in the recognition and removal of the damaged red cells or in the selective removal of the Heinz-bodies by the spleen. More recently, using immunofluorescence microscopy Heinz-body-induced clustering of Band 3 was detected in vivo in sickle cells [11] as well as in erythrocytes of patients with different unstable haemoglobin diseases [12]. Moreover, Schlüter and Drenckhahn [12] also demonstrated that the binding of autologous IgG to the surface of red cells revealing naturally occurring Heinz-bodies showed topographically identical distribution to that of the Band 3 clusters.

Because of its pathophysiological significance we decided to study Heinz-body-membrane interaction by electron microscopy. Since Band 3 is known to be a constituent of the intramembrane particles [13,14], the intense fluorescence over the phenylhydrazine-induced Heinz-bodies attributed to Band 3 clustering [10] would imply a marked aggregation of the intramembrane particles. Therefore, the aim of our study was to check Heinz-body-induced Band 3 clustering by freeze-fracture electron microscopy.

As Heinz-bodies cannot be visualized by freeze-fracturing in glutaraldehyde-fixed red cells (unpublished observation), phenylhydrazine-treated erythrocytes were frozen unfixed either by fast freezing in liquid propane or by conventional

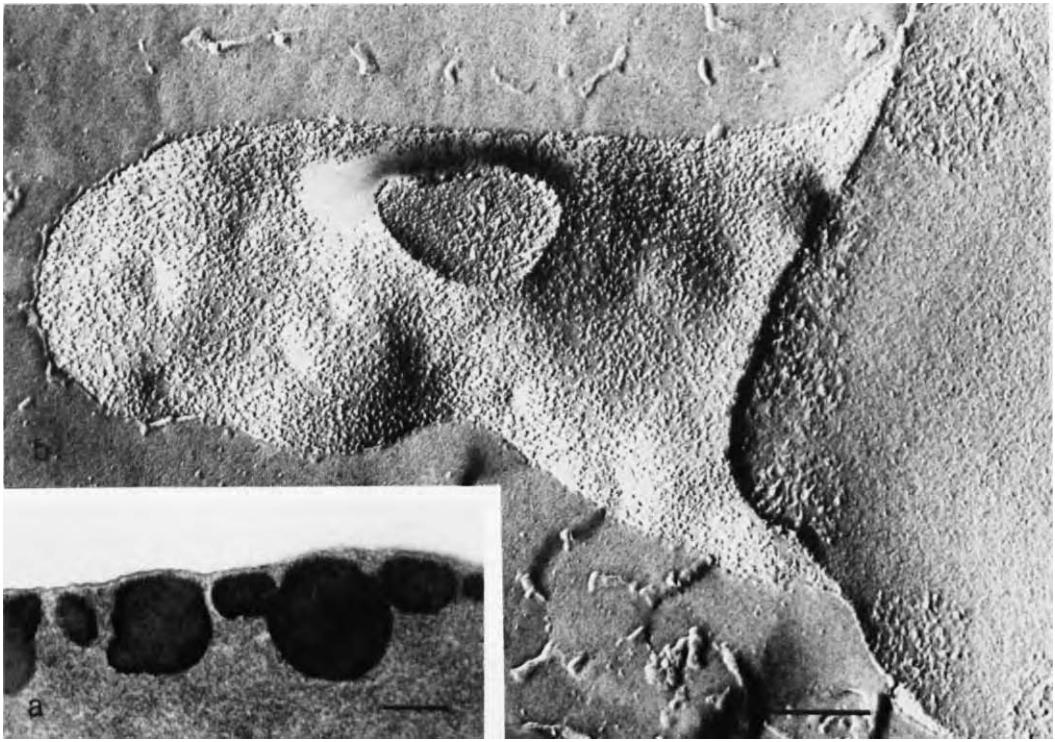

Fig. 1. Thin-section (a) and freeze-fracture (b) electron micrographs of washed, human erythrocytes treated with 15 mM phenylhydrazine hydrochloride for 2 h as described previously [10]. For thin-sectioning red cells were fixed in glutaraldehyde and osmium tetroxide followed by dehydration in graded ethanol and embedding in Durcupan ACM. For freeze-fracturing unfixed non-glycerinated erythrocytes were rapidly frozen in liquid propane using a Reichert-Jung KF 80 type apparatus followed by fracturing and platinum-carbon replication in a Balzers apparatus. (a) Observe the contact between the Heinz-bodies and the plasma membrane. Magnification, 42000×. Bar denotes 0.25 μm. (b) No clustering of the intramembrane particles can be seen either above the membrane-bound Heinz-bodies or in the remaining membrane areas. Magnification, 60000×. Bar denotes 0.25 μm.

freezing in Freon after glycerination. Fig. 1 shows phenylhydrazine-treated human erythrocytes prepared by thin sectioning and freeze-fracturing after fast freezing, respectively. Although the red cells were incubated in 15 mM phenylhydrazine for 2 h, which was found to induce marked Heinz-body formation and Band 3 clustering by Low et al. [10], we could not observe any change in the random distribution of the intramembrane particles (Fig. 1b). The same result was obtained when phenylhydrazine-treated, unfixed, glycerinated erythrocytes were freeze-fractured after conventional freezing (data not shown).

In trying to discover the reason for the striking contradiction between the results of Low et al. [10] and ours, we found that erythrocytes treated with phenylhydrazine as described above show an intense fluorescence when investigated by fluorescence microscopy (Fig. 2b). The observed fluorescence is not restricted to a given excitation range: it is orange-red in green, yellowish-green in blue, green in violet and bluish-green in ultraviolet excitation ranges. Lower concentrations of phenylhydrazine (below 5 mM) caused fainter and rapidly fading fluorescence. High concentrations of phenylhydrazine (above 5 mM) caused a considerable degree of haemolysis. In these cases the ghosts showed fluorescence localized exclusively to the membrane-bound Heinz-bodies (Figs. 2a and 2b). In the non-haemolysed red cells both the cytoplasm and the Heinz-bodies fluoresced, but the latter revealed a more intense fluorescence (data not shown). Sodium nitrite (which induces methaemoglobin but not Heinz-body formation) did not cause fluorescence even at concentrations higher than 15 mM. On the other hand, two other Heinz-body-inducing drugs acetylphenylhydrazine and hydrazine caused similar fluorescence at 15 mM concentration. The latter finding indicates that the presence of phenyl group is not necessary for the oxidant-induced red cell fluorescence. Moreover, since hydroxylamine (which is also a potent Heinz-body-inducing agent) caused similar fluorescence at concentrations above 5 mM after 4 h of incubation, the possibility that the fluorescence is due to the reported interaction between methaemoglobin and oxidized phenylhydrazine, phenyldiazene [6,15,16] can also be excluded. It should be mentioned that similar autofluorescence of Heinz-body-containing erythrocytes has already been described in haemoglobin Köln disease as well as in some other forms of unstable haemoglobin diseases [17,12]. Eisinger et al. [17] suggested that in the case of haemoglobin Köln disease the observed autofluorescence is due to the formation of a so-called fluorescent yellow pigment as a result of oxidative degradation of heme. The question of whether the fluorescence observed upon treatment with high concentrations of different oxidants can be explained in a similar way remaines to be determined.

The contradiction between the results of Low et al. [10] and ours could in principle be explained by considering the possibility that in our experiments

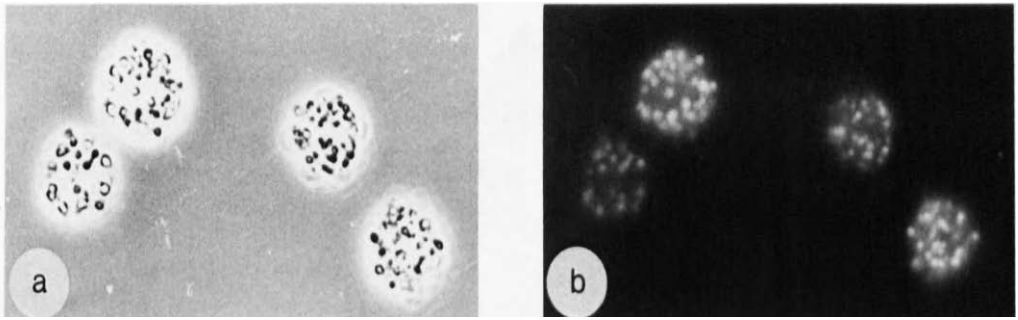

Fig. 2. Phase-contrast (a) and fluorescence (b) photomicrographs of the same erythrocytes treated with 15 mM phenylhydrazine hydrochloride as indicated under Fig. 1. The cells were washed four times after phenylhydrazine treatment and viewed unfixed in an Opton Photomicroscope III type microscope equipped with epifluorescence condenser and phase contrast optics. Note the intense fluorescence confined to the Heinz-bodies. The emitted light is orange-red when BP 515-560/LP 590, yellowish-green when BP 450-490/LP 520, green when BP 400-440/LP 470 and bluish-green when G 365/LP 420 type filter is used. Magnification, 1600×.

the Heinz-bodies formed initially in the cytoplasm and only after precipitation migrated to the membrane. In this case as pointed out by Waugh et al. [11] no clustering of Band 3 can be expected. We used exactly the same experimental procedures for red cell separation and Heinz-body formation as were employed by Low et al. [10]. Therefore, although the above-mentioned assumption may provide explanation for the lack of particle aggregation in our experiments, it cannot answer the question of why strong Band 3 clustering was observed by Low et al. under the same conditions.

In another experiment we compared the intensity of the phenylhydrazine-induced fluorescence detected at the excitation range of rhodamine to that of the specific immunofluorescence of red cells in which an intracellular antigen spectrin was detected using rabbit antihuman spectrin and rhodamine-conjugated goat antirabbit IgG. While in recent experiments performed on haemoglobinopathic erythrocytes [11,12] the red cell autofluorescence was found to be negligible compared to the specific immunofluorescence, our experiment (where photography and printing of both samples were also carried out identically) showed that the intensity of phenylhydrazine-induced fluorescence is in fact comparable to that of the specific immunofluorescence (data not shown).

Low et al. [10] used rhodamine-labelled antibody against the cytoplasmic segment of Band 3 to localize Band 3 distribution. Since the phenylhydrazine-induced fluorescence mimics the fluorescence characteristics of rhodamine, we think that this must have led to the authors' conclusion that Band 3 is significantly clustered above membrane-bound Heinz-bodies. Our freeze-fracture analysis suggests that this is not the case (Fig. 1b). Therefore, even if haemichromes precipitate Band 3 in solution [5], it is very unlikely, that binding of phenylhydrazine-induced haemichromes to the red cell membrane can cause massive Band 3 clustering in situ. It follows, furthermore, that the increased binding of autologous IgG to phenylhydrazine-treated red cells cannot be explained by strong, morphologically detectable Band 3 clustering [10]. However, we cannot exclude the possibility, that oligomerization of Band 3 may occur in phenylhydrazine-treated red cells over the Heinz-bodies, which can be detected biochemically, but not by freeze-fracturing. Such oligomerization, as were suggested by Low et al. [10], may in principle constitute the binding site for the autologous IgG. The possible colocalization of Heinz-bodies and surface-bound IgG which was detected by using fluorescein-labelled anti-human IgG [10] can only be confirmed or disproved by using morphological techniques other than fluorescence microscopy. Similarly, it is beyond the scope of this work to deal with Heinz-body-induced clustering of Band 3 detected by light microscopy in erythrocytes of patients with different haemoglobinopathies [11,12]. Using freeze-fracture and immunocytochemical electron microscopy these questions will be studied later on.

In the next set of experiments we studied whether membrane-bound Heinz-bodies have any effect on the aggregatability of the intramembrane particles. Phenylhydrazine-treated red cells were incubated in Acridine orange which is known to induce significant particle aggregation in intact red cells [18,19]. Since phenylhydrazine caused marked haemolysis at 15 mM concentration which was even more pronounced after the addition of the dye, Acridine orange treatment was performed on erythrocytes which had previously been incubated in 1.5 mM phenylhydrazine for 1 h. This lower concentration of phenylhydrazine was also found to induce a great number of membrane-bound Heinz-bodies [20]. Acridine orange treatment induced rapid spherocytic shape transformation and caused the membrane-bound Heinz-bodies to bud off from the erythrocytes after a few minutes of incubation. However, 1 minute after the addition of the dye most of the Heinz-bodies were still inside the red cells causing high protrusions on the plasma membrane (Figs. 3a and 3b). Phenylhydrazine treatment cannot prevent Acridine orange-induced particle aggregation either above the membrane-bound Heinz-bodies or in the intervening areas. About 50% of the protruded membrane areas over the Heinz-bodies showed the same degree of aggregation as was seen in the intervening areas, while the other half of these areas showed a significantly decreased particle aggregation relative to the remaining parts of the plasma membrane (Fig. 3b). The fact that Heinz-bodies cannot prevent particle aggregation indicates that there is no strong, covalent interaction

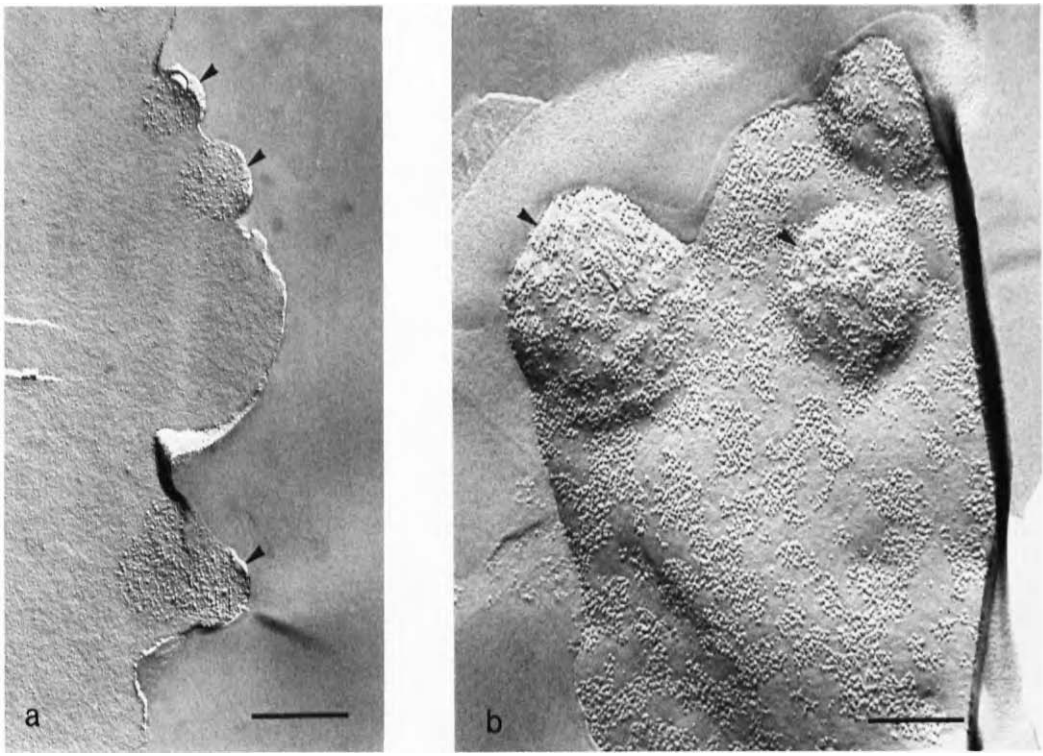

Fig. 3. Freeze-fracture electron micrographs of phenylhydrazine-treated erythrocytes incubated in Acridine orange. Red cells were washed in saline and diluted to 5% in phosphate-buffered saline (pH 7.4) containing 1.5 mM phenylhydrazine hydrochloride. Incubation was carried out for 1 h at 37°C. The erythrocytes were then washed and incubated for 45 min at 37°C in phosphate-buffered saline (pH 7.4) containing 10% glycerol. The cells were pelletted and 25 μl aliquots were added to 1 ml of Tris-buffered saline (pH 7.4) containing 2 mM Acridine orange and 10% glycerol. Conventional freezing was done 1 min after the addition of the dye. (a) Arrowheads indicate membrane-bound Heinz-bodies causing high protrusions of the plasma membrane. Magnification, 30000×. Bar denotes 0.5 μm. (b) Observe the aggregation of the intramembrane particles which is less pronounced over the Heinz-body-induced protrusions (arrowheads). Magnification, 60000×. Bar denotes 0.25 μm.

between the Heinz-bodies and all of the overlying Band 3 molecules. This is consistent with previously published data [5,21]. The reduction of Acridine orange-induced particle aggregation over numerous Heinz-bodies may reflect a true decrease in the local lateral mobility of the integral membrane proteins due to protein–protein (haemichrome–Band 3) interaction. However, it may also indicate that the solidification of anionic phospholipids upon treatment with Acridine orange is reduced as a result of an interaction between Heinz-bodies and lipids. Acridine orange-induced particle aggregation is most likely due to phase separation of anionic phospholipids in the red cell membrane [19]. Answering the question of how Heinz-bodies reduce particle aggregation requires further investigation.

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