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RECEPTOR DISTRIBUTION AND THE MECHANISM OF ENHANCED ERYTHROCYTE AGGLUTINATION BY SOYBEAN AGGLUTININ

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SUMMARY

We have examined the role of receptor clustering in intact erythrocyte membranes exhibiting enhanced lectin-mediated cell agglutination by analyzing freeze-fracture and freeze-etch images of human erythrocytes labeled with ferritin-conjugated soybean agglutinin. We find that trypsinization and fixation of intact erythrocytes, in either order, causes no alteration of the random distribution of ferritin-conjugated soybean agglutinin on the surfaces of these cells as compared to their distribution on the surfaces of fixed erythrocytes and untreated erythrocyte ghosts. Furthermore, clustering of the intramembranous particles in the membrane of intact erythrocytes was not found with any of the cells described above.

We conclude that clustering of the soybean agglutinin receptors is not a major factor involved in the enhanced agglutination of intact trypsinized erythrocytes. Caution is necessary in transferring information obtained with erythrocyte ghosts, where clustering can be induced, to intact erythrocytes.

INTRODUCTION

A current view is that increased lectin-mediated cellular agglutinability following trypsinization or transformation of cells results from an increased mobility and/or clustering of lectin receptors in the plane of the membrane. This suggestion is supported by the finding that trypsinization causes clustering of lectin receptors in 3T3 fibroblasts [1] and in erythrocyte ghosts [2] and that less agglutinable cells generally have a less clustered (i.e., a more random) distribution of receptors [3–10].

Recently we have shown that glutaraldehyde fixation of trypsinized human erythrocytes does not alter the trypsin enhanced agglutinability of these cells by lectins and more importantly, trypsinization of fixed erythrocytes increases their agglutinability to the level of trypsinized erythrocytes which have not been fixed [11]. Yet, the possibility existed that erythrocyte membranes fixed with glutaraldehyde might still retain mobile receptors which could cluster. Hence, it is of interest to investigate the actual topographic distribution of lectin receptors on the surfaces of these cells to provide direct insight into the role of receptor clustering in the agglutination reaction.

If the lateral mobility of the receptor sites is restricted by glutaraldehyde fixation of erythrocytes [12], then a trypsin mediated alteration of the distribution of binding sites for soybean agglutinin followed by glutaraldehyde fixation might be expected to leave the sites in their newly altered positions. In all cases using intact erythrocytes, the distribution of the labeled lectin and intramembranous particles was random, i.e., relatively dispersed, and the temporal order of trypsinization and fixation had no effect on this random pattern. These findings provide direct evidence that enhanced lectin mediated agglutination of erythrocytes does not depend on the clustering of the lectin receptor sites, i.e., that receptor mobility giving cluster formation is not the major factor involved in enhanced agglutinability of trypsinized cells.

MATERIALS AND METHODS

Red blood cells were obtained from fresh or outdated human whole blood anticoagulated with acid/citrate/dextrose. Erythrocyte ghosts were prepared by osmotic lysis according to the Dodge procedure [13]. Trypsinization was done with 0.1 mg of crystalline trypsin (Sigma) per ml of 4 % (v/v) erythrocyte or erythrocyte ghost suspension in phosphate buffered saline for 90 min at 37 °C.

Glutaraldehyde was used as supplied (Polysciences, 8 % in water under nitrogen, electron microscopic grade) or the glutaraldehyde (Baker, 25 % in water, practical grade) was distilled at atmospheric pressure. The distillate was stored at 4 °C as a 10 % solution in water. Erythrocytes and erythrocyte ghosts were fixed for 4 h at room temperature (20–22 °C) with constant agitation as 10 % suspensions in 2.0 or 2.5 % glutaraldehyde in phosphate-buffered saline as previously described [11]. Unreacted glutaraldehyde was removed routinely by 4 washes with phosphate-buffered saline.

Soybean agglutinin was purified by affinity chromatography as previously described [14].

Ferritin conjugation of soybean agglutinin was based on the glutaraldehyde-coupling method of Avrameas [15] and was almost identical to the procedure of Tillack et al. [2]. 3 ml of 50 mg/ml 6 times crystallized ferritin (Miles Pentex) was mixed with 50 mg of soybean agglutinin in 7 ml of 50 mM phosphate buffer, pH 7.0. While stirring this mixture at room temperature, 0.8 ml of neutral 0.5 % glutaraldehyde (Fischer) was added dropwise. The final mixture was constantly stirred at room temperature for 1 h. The mixture was dialyzed against 0.1 M NH_4Cl in 50 mM phosphate buffer, pH 7.0, for 5 h at 4 °C and then overnight against the phosphate buffer alone. The mixture was centrifuged at $10\,000 \times g$ for 15 min using a Sorvall RC2-B refrigerated centrifuge (10 °C); the pellet was discarded. The supernatant was applied to 2.5×100 cm agarose A 1.5 M (Biorad 50–100 mesh) column and eluted with 50 mM phosphate buffer, pH 7.0. The excluded peak contained the ferritin-soybean agglutinin conjugate as determined by Microtiter® assay of agglutination with fixed erythrocytes and by absorbance at 440 nm. Included volumes contained free ferritin and free soybean agglutinin. The conjugate was concentrated five-fold with an XM300 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The conjugation was done in the presence of 0.1 M D-galactose (Pfanstiel) which was subsequently removed by extensive dialysis. Agglutination activity of the conjugate was maintained.

The three types of fixed erythrocytes (fixed, fixed-trypsinized and trypsinized-fixed) and erythrocyte ghosts were each suspended as 10 % suspensions in isotonic phosphate buffer, pH 7.4, with equal volumes of ferritin-conjugated soybean agglutinin. The final concentration of soybean agglutinin was approximately 0.2 mg/ml so that agglutination of cells was quite apparent by visual inspection and by Microtiter® assay [11]. The aggregated cells or ghosts were then agitated and washed three times with approximate tenfold volumes of 5 mosM phosphate buffer. Centrifugations were done at $1000 \times g$ for 2 min for erythrocytes and at $20\,000 \times g$ for 10 min for erythrocyte ghosts, in a Sorvall RC2-B centrifuge. A final slurry of the cells or ghosts was prepared by aspirating off most of the supernatant buffer and resuspending the pellet. Small droplets of the cells or ghosts to be examined with the electron microscope were placed on copper planchets and frozen in liquid freon 12 cooled in liquid nitrogen to ~ −150 °C. The specimens were freeze-cleaved and etched for 3 min at − 100 °C and then shadowed with platinum-carbon in a Balzers freeze-etching apparatus. The platinum-carbon replicas were floated off the specimens on dilute buffer and bleach, cleaned with 40 % chromic acid and washed three times in distilled water. The replicas were then picked up on 200 or 400 mesh electron microscope grids and examined with a Philips 200 electron microscope.

RESULTS

The distributions of the intramembranous particles on the fracture face and the ferritin-conjugated soybean agglutinin on the external surfaces of erythrocyte ghosts (Fig. 1) are relatively random (i.e., dispersed, see ref. 16). A similar picture is obtained with fixed intact erythrocytes (Fig. 2A). The distributions found on the surfaces of erythrocytes that were trypsinized before fixation and erythrocytes that

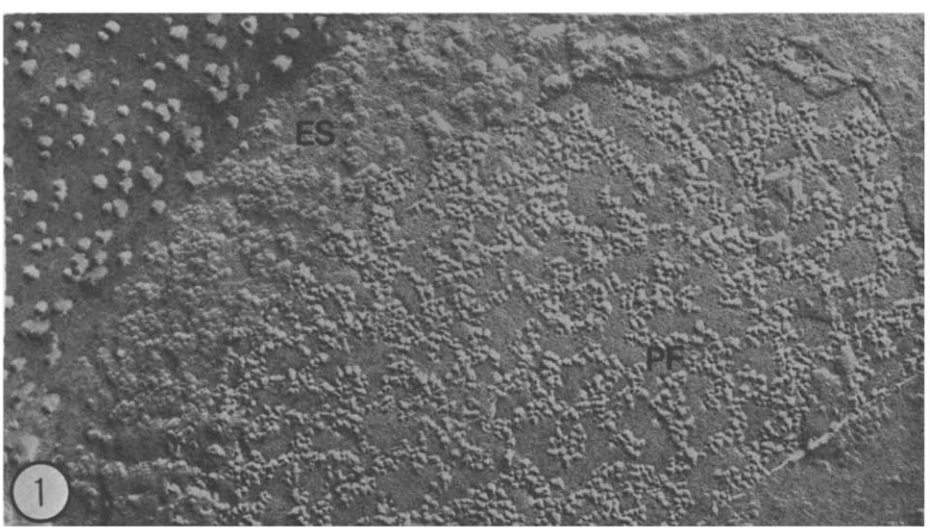

Fig. 1. Untreated erythrocyte ghost with relatively random distribution of ferritin-soybean agglutinin on etched surface (ES). A small amount of clustering of intramembranous particles on fracture face (PF) has occurred due to lysis and washing procedures. (× 76 000).

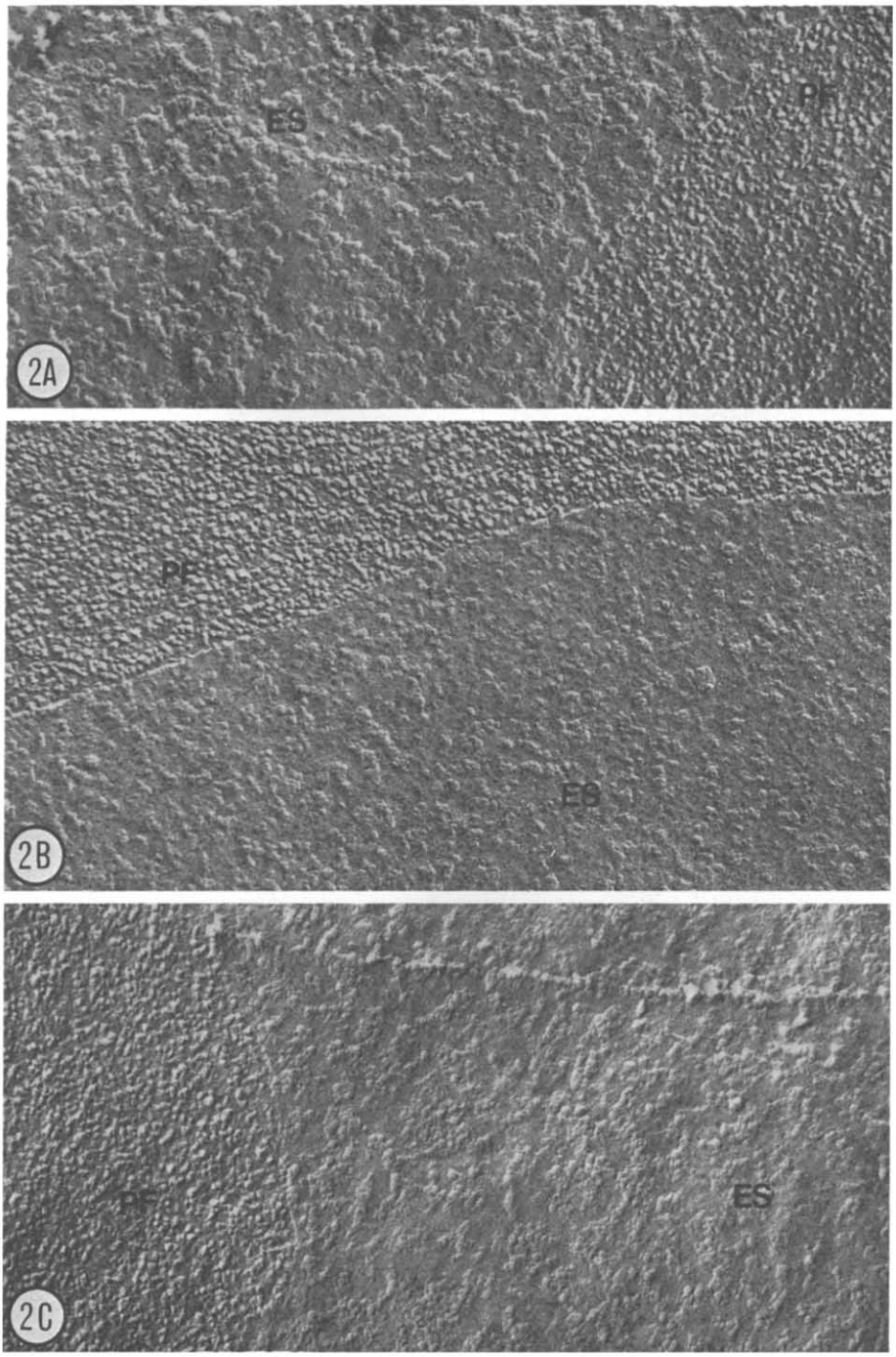

Fig. 2. (a) Fixed intact erythrocyte with random distributions of ferritin-soybean agglutinin and intramembranous particles. (×76 000). (b) Trypsinized-fixed intact erythrocyte with identical distributions of ferritin-soybean agglutination and intramembranous particles as in fixed erythrocyte. (76 000). (c) Fixed-trypsinized intact erythrocyte with random distributions of ferritin-soybean agglutinin and intramembranous particles. (×76 000).

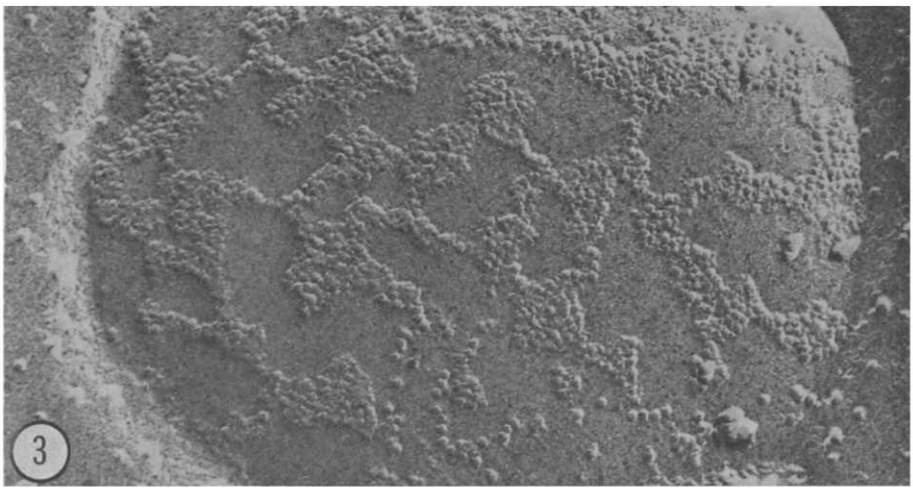

Fig. 3. Trypsinized-fixed erythrocyte ghost with ferritin-soybean agglutinin clustered on etched surface. (×88 000).

were trypsinized after fixation are indistinguishable from erythrocytes only pre-fixed (Fig. 2B, C)

It ghosts were trypsinized and subsequently fixed and labeled with the ferritin bound lectin, then considerable clustering of the ferritin-labeled soybean agglutinin was found to occur (Fig. 3). If ghosts were fixed prior to trypsinization, clustering still occurred (not shown). Overnight incubation in 20 mosM phosphate buffer, pH 5.5, followed by labeling with the ferritin conjugate resulted in a similar clustering of lectin and intercalated particles as reported by others under similar conditions [17].

The addition of 100 mM D-galactose, a specific inhibitor of soybean agglutinin, resulted in smooth external surfaces when added to incubation mixtures containing the ferritin-labeled lectin.

DISCUSSION

The clustering of lectin receptors has been implicated as the mechanism accounting for increased agglutinability of trypsinized cells and transformed cells [3–10]. This clustering has been correlated with the greater fluidity of the membrane believed to exist in intact transformed cells [4, 9, 18]. We have previously reported that increased agglutinability following the trypsinization of fixed erythrocytes as determined by Microtiter® assay is identical to trypsinized erythrocytes which are not fixed. This result was not easily accounted for by the clustering of lectin receptors in these cells unless fixation did not restrict receptor mobility in erythrocytes [11]. We can now directly rule out clustering of receptors as a necessary factor in enhanced agglutination; examination of freeze-etch electron micrographs of trypsinized then fixed erythrocytes labeled with ferritin-conjugated soybean agglutinin demonstrates a random distribution of the ferritin bound soybean agglutinin on the etched surfaces of these cells and a similar distribution of the intramembranous particles in the internal matrix of these cells. Identical findings were made for fixed-then-trypsinized and

untrypsinized-fixed cells. The degree to which the mobility of lectin receptors has been restricted by glutaraldehyde fixation is not known. However, it is clear that the case of clustering of these receptors is not the essential factor accounting for the greater agglutinability of these cells.

This finding parallels some evidence that the distribution of intramembranous particles in transformed cells can be more random (less clustered) than in untransformed cells [19, 20] and that clustering may even reduce the extent of the agglutination reaction under certain conditions [21]. In addition, a recent study indicates that concanavalin A has a random distribution on the surfaces of native erythrocytes unless anti-concanavalin A antibody is subsequently added [22]. They conclude that clustering of receptor sites is unnecessary for agglutination.

We have also shown that the conditions which lead to clustering of receptors in erythrocyte ghosts do not cause clustering in the intact erythrocyte, in agreement with previous observations [23]. This difference is possibly due to loss from ghosts of the inner surface peripheral protein, spectrin, which possibly has links to the intramembranous particles [24, 25] and may restrain particle mobility in the intact erythrocyte [16]. Our observation dictates caution in transferring information obtained from ghosts to intact erythrocytes.

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