CONCANAVALIN A-INDUCED ENDOCYTOSIS IN RABBIT RETICULOCYTES, AND ITS DECREASE WITH RETICULOCYTE MATURATION

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ABSTRACT

Concanavalin A (Con A) was taken up to a limited extent by endocytosis in rabbit reticulocytes but not in rabbit erythrocytes. This process was observed by the use of ferritin-labeled Con A and transmission electron microscopy of thin sections of plastic-embedded cells. Furthermore, the extent of endocytosis among the reticulocytes decreased with the extent of their maturation, reticulocyte age being measured by ribosome configurations. These results are consistent with the proposal that there are domains in the membranes of reticulocytes in which the Con A receptors are laterally mobile, and can be clustered and endocytosed. These mobile domains exist, or are formed, within a larger framework of immobile membrane. During reticulocyte maturation, these domains are gradually eliminated, eventually disappearing upon formation of the mature erythrocyte. Possible molecular mechanisms for this proposed elimination process are discussed.

KEY WORDS membrane fluidity · mobile domains · erythrocyte maturation · spectrin complex

It has been realized for some time that the membranes of mature mammalian erythrocytes are unusual in that their integral proteins generally appear to be severely restricted in their lateral mobility in the plane of the membrane (12, 7, 19). When antibodies or lectins are bound to their specific receptors on the surfaces of lymphocytes, for example, they generally induce a cross-linking, followed by a patching, capping, and endocytosis of their receptors in the fluid membrane (for a review, see reference 20). By contrast, when the same kinds of experiments are carried out with adult human erythrocytes, no indication of a redistribution of receptors is observed under physiological conditions (12). The lipid matrix of the erythrocyte membrane is sufficiently fluid so that the lipid viscosity of the membrane cannot be responsible for the apparent lateral immobility of its receptors. Instead, it is thought that the spectrin complex (cf. references 1, 7, 22, and 23) forms a "scaffolding" under the membrane which is somehow responsible for the immobilization of receptors, but there is no agreement about the precise mechanism involved.

The mature erythrocyte is the end stage of erythroid cell differentiation, the previous stage of which is the reticulocyte. Schekman and Singer (19) reported that ferritin-conjugated concanavalin A (F-Con A) is to some extent clustered and endocytosed after binding to neonatal human erythrocytes (and not adult human erythrocytes) but to a much greater extent with neonatal human reticulocytes. To explore this interesting difference between reticulocytes and erythrocytes more directly, we have turned to a comparison of the response of erythrocytes and reticulocytes of rabbits to F-Con A treatment, because standard methods, particularly phenylhydrazine-induced hemolytic anemia, are available to enrich greatly the circulating reticulocyte population in rabbits. In this communication, we show not only that rabbit reticulocytes (but not erythrocytes) exhibit

a clustering and endocytosis of F-Con A, but that their capacity to do so decreases progressively as the reticulocytes mature.

MATERIALS AND METHODS

Phenylhydrazine hydrochloride was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co. (Rochester, N. Y.) and recrystallized from 1 N HCl before use. Rabbits weighing ~2.5 kg were injected subcutaneously for 4 consecutive days with 1 ml of 30 mg/ml phenylhydrazine in physiologic saline. 1 day after the last injection, blood was obtained by intracardiac puncture into heparinized syringes. The blood from one rabbit was used for each series of experiments, although the results showed little variance between rabbits.

F-Con A was prepared by a modification of the method of Avrameas, as previously described (14).

With all operations performed at 4°C, the cells were washed once by centrifugation in 0.09 M phosphate-buffered saline, pH 7.4, and resuspended in 1,000 vol (0.1% cells) of the buffer. This extreme dilution is necessary to prevent the Con A from subsequently agglutinating the cells. F-Con A was added to a final concentration of 1 μg of Con A per ml of cell suspension. The samples were transferred to a 37°C waterbath and incubated for 30 min. Incubation was terminated by the addition of an equal volume of 2% glutaraldehyde (in the same buffer), and the cells were fixed for 2 h. The cells were then washed, postfixed in 2% osmium tetroxide, dehydrated, and embedded in Epon. Silver sections (~80 nm thick) were cut, stained with uranyl acetate and lead citrate, and examined in a Philips model 300 electron microscope.

RESULTS

Reticulocytes in the blood samples of rabbits with phenylhydrazine-induced anemia were readily recognized as enucleated electron-dense cells that contained ribosomes. "Young" reticulocytes were taken to be those with >50% of their ribosomes present as polyribosomes, and "old" reticulocytes those with >50% of their ribosomes as monosomes (6, 9, 13, 18). Almost all the reticulocytes had the bulk of their ribosomes in one configuration or the other. The old reticulocytes, in addition, generally contained Heintz bodies resulting from the phenylhydrazine treatment.

After the 30-min incubation at 37°C, F-Con A appeared clustered on invaginations of the membrane as well as inside the reticulocytes in the form of closed vesicles lined on their inner surfaces with ferritin particles. This is shown in Fig. 1. (That the vesicles were not simply deep surface invaginations was demonstrated in several cases by serial sectioning.) These vesicles varied in both size and the number of ferritin particles they contained. The small amount of ferritin that is naturally present in reticulocytes (24) was not a source of confusion, because the endogenous ferritin is either free in the cytoplasm or in morphologically distinct siderocytes. Free ferritin itself was not endocytosed when incubated with the reticulocytes. Mature rabbit erythrocytes did not exhibit either F-Con A-lined membrane invaginations or intracellular vesicles (Fig. 1 *c*).

Quantitative measures of the endocytosis were obtained by the following procedures: To avoid systematic differences in section thickness, all data were taken from a single large section. From a randomly designated area on this section, all cells were photographed. Each cell was classified as either a young reticulocyte, old reticulocyte, or erythrocyte. Of the 290 cells photographed, 155 were young reticulocytes, 85 were old reticulocytes, and 50 were erythrocytes. The number of ferritin-containing vesicles per cell were recorded along with the width of each ferritin vesicle at its longest dimension.

The results of this survey are shown in Figs. 2 and 3. The average number of ferritin vesicles per cell section declined from a high of 3.0 for young reticulocytes to 0.94 for old reticulocytes, and 0.02 for erythrocytes. A histogram of this distribution is shown in Fig. 2. Fig. 3 shows that the decrease in the numbers of ferritin vesicles with age was not uniform with respect to vesicle size. In particular, there was an 11-fold decrease in the number of ferritin vesicles with widths <120 nm from young to old reticulocytes. By contrast there was much less of a decrease in the number of the larger ferritin vesicles.

During the maturation process, the reticulocyte becomes smaller (2, 21). The average areas of old reticulocytes and erythrocytes were found to be 84 and 55% of that of the young reticulocytes, and the results in Fig. 3 have been normalized to express the data as they would be if old reticulocytes had the same size as young ones. All other data are expressed without this small correction.

DISCUSSION

The relative decline in polysomes and increase in monosomes associated with reticulocyte maturation in vivo has been well-documented (6, 9, 13, 15, 18), but rapid processing and fixation of the cells is important in preventing changes in the naturally occurring ribosome configurations (15). The absence of Heintz bodies in the majority of

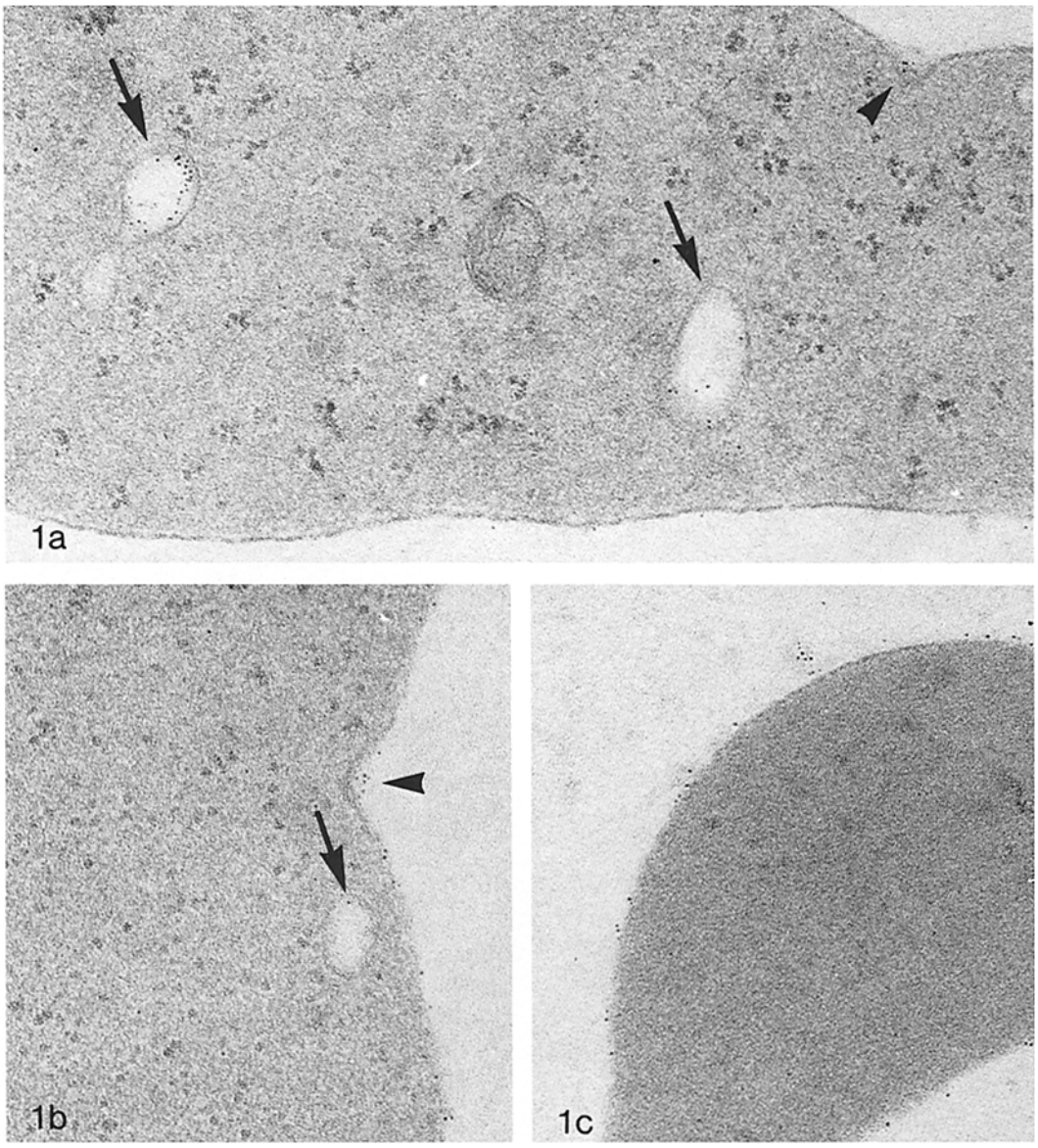

FIGURE 1 Electron micrographs of the ferritin-Con A-treated rabbit reticulocyte population. (*a*) Young reticulocyte with clustered ribosomes, ferritin vesicles (arrows), and a ferritin-lined invagination (arrowhead). (*b*) Old reticulocyte with monosomes, a single ferritin vesicle (arrow) and a ferritin-lined invagination (arrowhead). Although only one ferritin particle is present in the vesicle shown, no significant change with maturation in the average number of ferritin particles per vesicle was observed. (*c*) Erythrocyte showing no endocytotic vesicles or ferritin-lined invaginations, the ferritin-Con A distributed randomly on the cell surface. × 66,000.

the reticulocytes that contained mostly polyribosomes, and their presence in the cells that contained mostly monosomes, was a confirmation of reticulocyte age (17).

Our results indicate that the maturation from old to young reticulocytes to mature erythrocytes is accompanied by a progressive decrease in the extent to which the endocytosis of Con A membrane receptors occurs, until at the last state of the process, the mature erythrocyte exhibits no

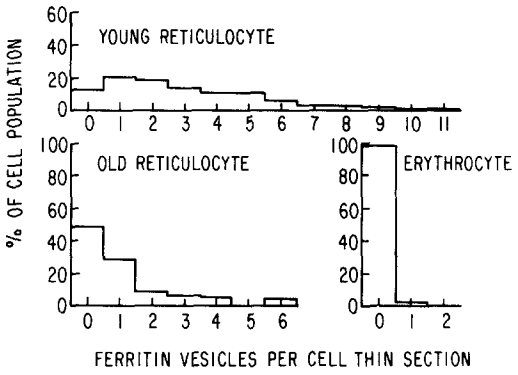

FIGURE 2 Histograms showing the distribution of ferritin vesicles throughout the cell population. The average number of ferritin vesicles per cell decreases during maturation.

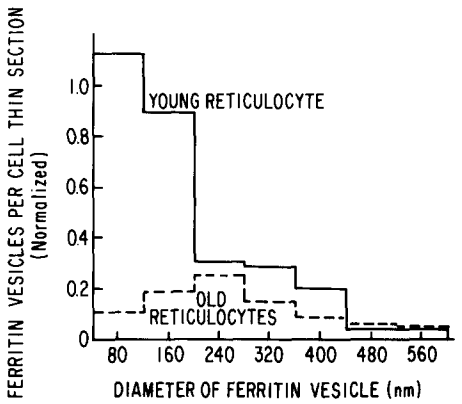

FIGURE 3 Histograms showing the distribution in the sizes of the ferritin vesicles for young and old reticulocytes. The large decrease in endocytosis is due primarily to a decrease in the formation of smaller vesicles as the reticulocyte matures. The results have been normalized to give old reticulocytes the same area in thin section as young reticulocytes (see text).

endocytosis. Schekman and Singer have shown (19) that such endocytosis requires the clustering of the Con A receptors in the membrane, and hence their lateral mobility in the plane of the membrane. The presence of clusters of F-Con A found on invaginations of the reticulocyte membrane whereas isolated F-Con A is sparsely and randomly distributed elsewhere on the membrane (Fig. 1 *a* and *b*), presumably reflects the collection of the Con A receptors into the regions of the membrane that become invaginated. The formation of invaginations is followed by their endocytosis. The progressive decrease in endocytosis with maturation is therefore most probably due to a progressive decrease in Con A receptor mobility in the reticulocyte membrane.

However, the limited nature of the Con A receptor mobility even in the membranes of young reticulocytes must be appreciated. Observations with fluorescein-conjugated Con A at the light microscope level of resolution with mammalian (12, and footnote 1) as well as avian reticulocytes and erythrocytes (3) yielded no clear indication of any patching, capping, or endocytosis of the Con A receptors. With F-Con A at the electron microscope level of resolution, however, the endocytosis of ferritin-lined vesicles can readily be observed, even if the majority of the F-Con A is left on the cell surface.

In related studies with neonatal human erythrocytes (19, and footnote 2), Schekman and Singer have interpreted such limited receptor mobility to be due to the presence initially, or to the ligand-induced formation, of discrete domains in the membrane within which integral proteins exhibit lateral mobility, with such domains dispersed within a much larger matrix of immobilized proteins. As the immobilization is thought to be due to a scaffolding of the spectrin complex attached to the cytoplasmic face of the membrane (cf. references 1, 7, 22, and 23), the mobile domains might reflect gaps or imperfections in that scaffolding. This proposal is supported by the findings in the preceding communication (25) that regions of the membranes of neonatal human erythrocytes and reticulocytes that become invaginated or endocytosed after incubation with Con A show an absence of immunoferritin staining for spectrin, whereas immediately contiguous regions of the membranes are heavily stained.

In terms of this hypothesis of mobile domains, therefore, we propose that the progressive decrease in Con A receptor mobility and endocytosis with reticulocyte maturation may be the result of the progressive elimination of the gaps or imperfections in the spectrin scaffolding. If gaps or imperfections had to exceed a certain critical size for them to function as a mobile domain, a uniform rate of sealing them might be expected to eliminate the smaller ones first. Alternatively, the mechanism of elimination may be more efficient

[1] Geiduschek, J., and S. J. Singer. Molecular changes in the membranes of mouse erythroid cells accompanying differentiation. *Cell*. In press.

[2] R. Schekman and S. J. Singer. Lectin and antibody-induced endocytosis in neonatal human erythroid cells. Manuscript in preparation.

for small gaps compared to large ones. Either of these possibilities could account for the disproportionate loss of smaller endocytotic vesicles as the reticulocyte aged (Fig. 3). As the synthesis of the spectrin complex no longer occurs in reticulocytes (11), the mechanism for elimination of gaps or imperfections in the spectrin scaffolding could not be the addition of spectrin to the membrane. Such mechanisms could, however, involve (*a*) membrane remodeling in vivo and (*b*) metabolic changes, accompanying maturation. The reticulocyte has a larger membrane surface area and volume than the mature erythrocyte. There is evidence that reticulocyte maturation involves a disproportionate loss of membrane compared to cell volume (4, 5, 8), this loss occurring by exocytotic and/or endocytotic mechanisms.[3] If the spectrin complex was depleted from those regions of the reticulocyte membrane that were lost during maturation, the complex would become increasingly concentrated on the residual membrane of the maturing cell. Such membrane remodeling could thus eliminate gaps or imperfections in the spectrin scaffolding. The structure and function of the spectrin complex, however, is very likely influenced not only by the concentration of spectrin but also by the metabolism of the cell. There is good evidence that the state of aggregation of the spectrin complex is at least partially regulated by the phosphorylation and dephosphorylation of spectrin (1, 16), and it is conceivable that the steady-state level of such phosphorylation might change progressively in the course of reticulocyte maturation. Such metabolic changes, therefore, might also contribute to structural changes in the spectrin scaffolding that progressively eliminated the mobile domains.

On the other hand, it is also possible that other changes in the reticulocyte membrane, e.g., the loss of a membrane protein (10), could be important to the changes in membrane properties with maturation in some as yet unknown ways.

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[3] Rabbit reticulocytes produced by phenylhydrazine treatment (so-called stress reticulocytes) are larger than those in the normal circulation. The membrane remodelling observed with the stress reticulocytes, however, probably applies as well to normal reticulocytes (4, 5).

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