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THE INTERACTION BETWEEN TRANSFERRIN AND RABBIT RETICULOCYTE GHOSTS

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SUMMARY

1. The properties of the membrane receptors involved in iron transfer from plasma transferrin to cells were studied using rabbit reticulocyte ghosts prepared by hypotonic haemolysis and pure rabbit plasma proteins labelled with ^{125}I, ^{131}I and/or ^{59}Fe.

2. The specificity of the transferrin-binding receptors was apparently lost in preparation of the reticulocyte ghosts. In contrast to the reaction with intact cells, transferrin uptake and reflux from ghosts was independent of the reticulocyte count, was similar in quantity and kinetics to albumin uptake and was not affected by sulphydryl reagents which inhibit transferrin uptake by intact cells.

3. These results indicate that the structural integrity of the reticulocyte is critical for the specific uptake of transferrin and that identification and isolation of transferrin receptors after hypotonic lysis is unlikely to be successful.

INTRODUCTION

During the process of iron exchange between the plasma iron transport protein, transferrin, and reticulocytes the transferrin becomes bound to the cell [1, 2]. It is thought that this binding occurs at specific receptors for transferrin on the reticulocyte cell membrane [3]. Little information is available on the nature or properties of these receptors. The present investigation was undertaken using rabbit transferrin and reticulocyte ghosts in order to study this problem prior to making attempts to isolate transferrin receptors from the reticulocyte cell membrane. It was presumed that the transferrin receptors would still be present in the cell stroma after preparation of the ghosts and that they would still react with transferrin. The elimination of the soluble components of the cell, especially haemoglobin, during haemolysis might allow for more facile isolation of transferrin receptors.

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MATERIALS AND METHODS

Chemicals

Reagents were of analytical grade when obtainable. Trypsin (Type 3), ATP, NADH and ascorbic acid were purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Trypsin inhibitor (from egg white) was obtained from Calbiochem, San Diego, Calif., U.S.A. ^{59}Fe ($FeCl_3$, 10–20 μCi/μg), ^{125}I and ^{131}I (NaI, carrier-free) were purchased from the Radiochemical Centre, Amersham, England.

Protein purification and labelling

Transferrin and albumin were prepared from rabbit serum and labelled with ^{131}I or ^{125}I, and, in the case of transferrin, with ^{59}Fe as described previously [4, 5].

Reticulocyte and ghost preparation

Reticulocyte-rich blood was obtained from rabbits made anaemic by bleeding from a marginal ear vein (15 ml/kg, three times weekly). Blood low in reticulocytes was obtained from previously untreated rabbits. In the initial experiments ghosts were prepared from the erythrocytes of bled or unbled rabbits by mixing the packed, washed cells with 15 vol. of haemolysis solution for 5 min, either at 4 °C or at 37 °C. Two haemolysis solutions were used, viz. 20 mM sodium phosphate buffer, pH 7.4 and the same buffer containing 1 mM $CaCl_2$ added immediately before haemolysing the cells. After haemolysis the ghost preparations were resealed by the addition of sufficient 3 M KCl to achieve a final concentration of 0.160 M and by incubation at 37 °C for 15 min [6]. Following haemolysis and resealing the ghosts were separated from the haemolysate by centrifugation at 4 °C for 20 min at $4000 \times g$ and were washed three times by resuspension in Hanks and Wallace balanced salt solution [7] and centrifugation as before.

Incubation procedure

The procedures used for measuring the uptake by or release from the cells or ghosts of labelled protein were the same as in earlier experiments [8, 9]. In any one experiment, the cells or ghosts were incubated with transferrin doubly labelled with radioactive iodine and ^{59}Fe, or with transferrin and a second plasma protein. Where two proteins were used in any one experiment, they were labelled with different isotopes of iodine. The concentration of each protein in the incubation medium was 2 mg/ml. To compare the rate of reflux of labelled protein from cells or ghosts pre-incubated at 37 °C for 30 min, the suspensions were washed at 4 °C to remove unbound protein and re-incubated at 37 °C in Hanks solution. Aliquots were removed into 4 ml ice-cold 0.15 M NaCl at time intervals up to 100 min, centrifuged and protein reflux was estimated from the proportion of each label in the supernatant and cells or ghosts.

Analytical methods

Iron concentrations and total iron-binding capacity of transferrin-containing solutions were measured using the method of Morgan and Carter [10]. Protein was determined by a buiret procedure [11]. Radioactivity was measured in a dual-channel well-type scintillation counter. Reticulocytes were counted on dry smears after staining with new methylene blue.

RESULTS

Iron uptake

As has been described previously [2, 12–14], iron uptake by intact reticulocytes was found to increase steadily with time of incubation at 37 °C, but uptake by reticulocyte ghosts was very low and could be attributed entirely to iron bound to transferrin taken up by the ghosts.

Protein uptake

Transferrin and albumin were taken up by reticulocyte ghosts as well as by intact cells but the pattern of results obtained with ghosts was different from that of intact cells (Fig. 1). In intact cells transferrin uptake was far greater than that of albumin, and transferrin uptake occurred in a biphasic manner, initial rapid binding or adsorption being followed by a slower, progressive uptake during the succeeding 5–15 min of incubation at 37 °C. This second phase of uptake has been called the association reaction [8]. Albumin uptake by intact reticulocytes showed little increase during the period of incubation. By contrast with intact cells the uptake of both transferrin and albumin by ghosts increased progressively during incubation at 37 °C for up to 30 min so that there was a much greater uptake of albumin by ghosts than by intact cells.

The effect of preparing the ghosts in different ways is shown in Fig. 1. Generally, the pattern of transferrin and albumin uptake was similar with the different methods used but there were quantitative differences in the amounts of proteins taken up. The presence of Ca^{2+} in the haemolysing solution resulted in an increase in uptake of both proteins when the ghosts were subsequently incubated with them. Similarly

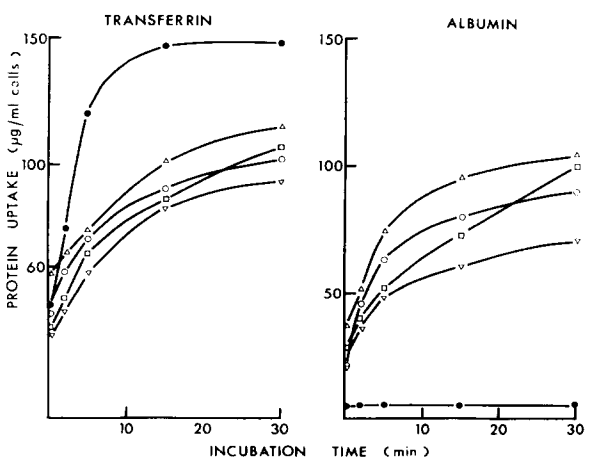

Fig. 1. Uptake of rabbit transferrin and albumin at 37 °C by rabbit reticulocytes (●) and reticulocyte ghosts (○, △, ▽, □). The ghosts were prepared by haemolysing the cells in 20 mM sodium phosphate buffer (pH 7.4) at 4 °C (△, ○) or at 37 °C (□, ▽) with (△, □) or without (○, ▽) the addition of 1 mM $CaCl_2$ to the haemolysing buffer. The reticulocyte count was 30 % and the concentrations of both proteins in the incubation solution were 2 mg/ml.

haemolysis at 4 °C resulted in greater uptake of the proteins than did haemolysis at 37 °C.

In all subsequent experiments the method of haemolysis used was to mix the washed cells with 20 mM phosphate buffer containing 1 mM $CaCl_2$ at 4 °C for 5 min. In certain experiments, as indicated below, other substances were added to the haemolysis solution. Following haemolysis the ghosts were resealed and washed as described in Materials and Methods.

Another difference between intact cells and ghosts which was found was the effect of reticulocyte count on the uptake of transferrin. As shown in Fig. 2 transferrin uptake by intact cells after incubation at 37 or at 4 °C was directly proportional to the reticulocyte count. However, with ghosts the reticulocyte count had only a slight effect on transferrin uptake. The uptake of albumin by either cells or ghosts was not affected by reticulocyte count.

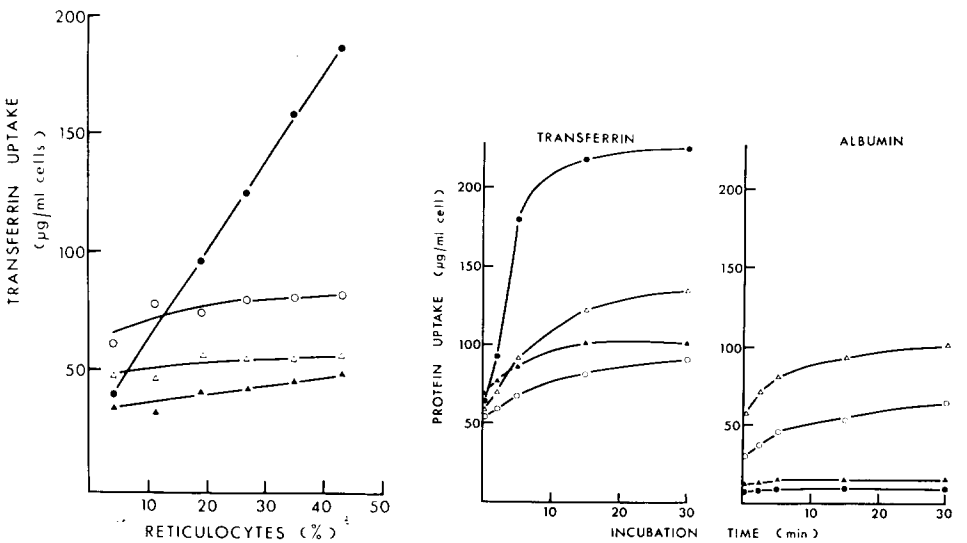

Fig. 2. Effect of reticulocyte concentration on the uptake of transferrin by intact cells (●, ▲) or their ghosts (○, △) when incubated with ^{125}I-labelled transferrin (2 mg/ml) at 4 °C (▲, △) or at 37 °C (●, ○) for 30 min.

Fig. 3. Effect of pH on the uptake of transferrin and albumin by reticulocytes and their ghosts. The cells (●, ▲) or ghosts (○, △) were pre-incubated for 20 min at 37 °C in 310 mM sodium phosphate buffer, pH 7.4 (●, ○) or pH 5.5 (▲, △), prior to addition of the labelled proteins (concentrations, 2 mg/ml). The reticulocyte count was 37 %.

Effect of pH, tryptic digestion, ATP and sulphydryl reagents on protein uptake

Reticulocytes and their ghosts were preincubated for 20 min at 37 °C in 310 mM sodium phosphate buffer at either pH 7.4 or 5.5. Radioactive-labelled transferrin and albumin in the same buffers were then added and incubation was continued, samples being taken at different times after adding the labelled proteins. As shown in Fig. 3 the uptake of transferrin by intact reticulocytes was much lower at pH 5.5 than at 7.4, while albumin uptake was greater at the lower pH. In the case of ghosts, however, the uptake of both proteins was greater at pH 5.5 than at 7.4.

The effect of tryptic digestion of the cells on transferrin and albumin uptake was studied by incubating washed reticulocytes with trypsin (0.5 mg/ml in 20 mM sodium phosphate–135 mM NaCl) for 15 min, adding a 4-fold excess of egg-white trypsin inhibitor, washing the cells twice with 0.15 M NaCl and preparing ghosts from half of the treated cells. In the case of intact cells tryptic digestion produced approximately 65 % reduction in transferrin taken up at 0 min and at subsequent times of incubation; albumin uptake, on the other hand, was approximately doubled. With ghosts there was a 50 % reduction in the uptake of both transferrin and albumin from the control values.

When 2 mM Mg^{2+}-ATP was included in the haemolysis solution and the ghosts then resealed and washed prior to incubation with transferrin and albumin, the uptake of the proteins was found to increase, both by approximately 35 %. The ghosts produced in the presence of ATP retained more of their haemoglobin and were distinctly red in colour compared with the control ghosts which had only a slight pink colour.

It has previously been shown that several metabolic inhibitors which react with sulphydryl groups block the progressive uptake of transferrin by reticulocytes but have no effect on albumin uptake [9]. The effects of three such inhibitors (*N*-ethylmaleimide, sodium arsenite and iodoacetamide) on transferrin and albumin uptake by reticulocytes and their ghosts was therefore investigated. The results are summarized in Table I. Whereas the three inhibitors markedly affected transferrin uptake by intact reticulocytes they had no effect on transferrin or albumin uptake by ghosts.

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE, SODIUM ARSENITE AND IODOACETAMIDE ON TRANSFERRIN AND ALBUMIN UPTAKE BY RABBIT RETICULOCYTES AND THEIR GHOSTS

The results are expressed as a percentage of the value for uptake of labelled protein by control samples of cells or ghosts incubated in the absence of inhibitors. Each value is the mean of 4 measurements ±S.E. The concentration of reticulocytes varied from 20 to 35 %. The inhibitor concentrations were 10 mM. Total uptake was that determined after incubation at 37 °C for 30 min; 37 °C−0 °C uptake was the difference between total uptake and the uptake observed after incubation at 0 °C.

	Uptake (% control value)					
	Intact cells			Ghosts		
	N-Ethyl-maleimide	$NaAsO_2$	Iodo-acetamide	*N*-Ethyl-maleimide	$NaAsO_2$	Iodo-acetamide
Transferrin						
Total	31 ±0.9	38 ±1.9	34 ±0.6	101 ± 10.8	103 ± 3.3	101 ± 1.5
37 °C−0 °C	5.6 ± 2.3	9.8 ± 1.4	8.0 ± 1.4	106 ± 18.3	98 ± 7.2	104 ± 11.1
Albumin						
Total	101 ±1.3	99 ±0.9	102 ±2.5	106 ± 7.3	101 ± 1.3	119 ± 17.2
37 °C−0 °C	–	–	–	108 ± 9.2	96 ± 6.6	109 ± 10.3

Protein reflux

When reticulocytes or their ghosts were incubated with labelled transferrin and albumin for 30 min, washed and reincubated in Hanks' solution, the two labelled proteins were released into the reincubation medium. The release of both proteins was rapid initially, later slowing and was incomplete even after periods of incubation up to 100 min. Transferrin release from intact reticulocytes occurred more rapidly and to a much greater degree than did transferrin release from ghosts (Fig. 4). The pattern of albumin release, however, was similar for intact cells and ghosts and was comparable to that of transferrin release from ghosts.

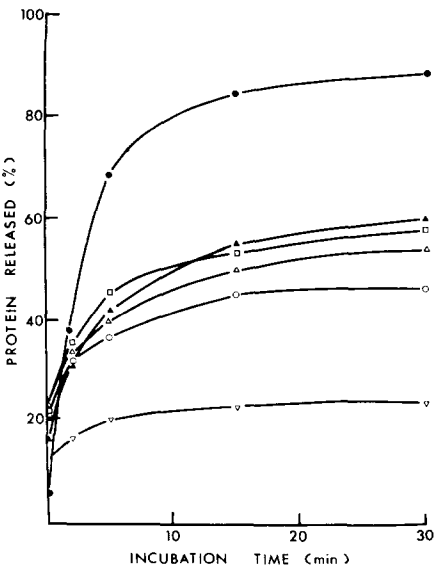

Fig. 4. Release of transferrin (●, ○, ▽) and albumin (▲, △, □) from rabbit reticulocytes (●, ▲) and their ghosts (○, ▽, △, □). The proteins released from the ghosts had either been taken up after their preparation from reticulocytes (○, △) or by intact cells prior to haemolysis and preparation of the ghosts (▽, □).

Two other methods of incorporating labelled transferrin and albumin into reticulocyte ghosts were used for comparison with that described above. In the first intact reticulocytes were incubated with the labelled proteins for 30 min, washed and ghosts were obtained in the standard manner. These ghosts were then reincubated in Hanks' solution in order to measure the release of the labelled proteins. The results are shown in Fig. 4. Albumin release was similar to that described above. However, transferrin release was much less than that which occurred when ghosts had been used for the initial uptake step.

The second additional method of labelling ghosts which was used was adding the proteins to the haemolysis solution so that they were taken up during the process of haemolysis before resealing and washing. When this was done, the pattern of release of the proteins during subsequent reincubation was almost identical with that found when resealed ghosts had been used for uptake.

DISCUSSION

The results obtained in these experiments indicate that the structural integrity of the reticulocyte is critical in the specific interaction between transferrin and reticulocytes. Transferrin uptake by ghosts was apparently non-specific in that it was similar to that of albumin whereas with intact reticulocytes it was much greater than albumin uptake. Also, transferrin uptake by ghosts was not dependent on reticulocyte count to nearly the same degree as was uptake by intact cells. Furthermore, the rate of reflux of transferrin from reticulocyte ghosts was similar to that of albumin and much slower than reflux from intact cells. Finally the sulphydryl reagents had no effect on transferrin uptake by ghosts when used at concentrations which greatly inhibited uptake by intact cells, and alteration of the incubation pH or pre-treatment with trypsin produced different effects on the uptake of transferrin and albumin by intact cells, yet with ghosts their effects were in the same direction for the two proteins and were quantitatively of approximately the same degree.

It may be concluded that the process of hypotonic haemolysis produces changes in the reticulocyte resulting in a loss of ability to take up transferrin by the specific association reaction previously described [8, 9]. Other studies have shown that iron uptake by ghosts is negligible even when the ghosts are reconstituted with their normal intracellular constituents [2, 12–14]. The present investigation indicates that it is the loss of membrane integrity which blocks iron uptake by preventing the specific interaction of the iron carrier, transferrin, with the cell membrane.

The loss of specificity in the reaction with transferrin which occurs when reticulocytes are haemolysed suggests that a membrane component essential for the specific interaction between transferrin and the cell may be lost or altered during haemolysis. The outermost layer of the cell is a loose mucopolysaccharide region containing a number of strongly negatively charged groups [15] of which sialomucopeptides may be a dominant constituent [16]. This mucopeptide region is largely removed during the preparation of ghosts [17]. It may be critical in determining the specific interaction between transferrin and reticulocytes, although studies on sialic acid depleted reticulocytes [18] or polyamino acid treated reticulocytes [5] suggest that surface change is not important in the transferrin–reticulocyte interaction. An alternative explanation is that steric factors are critical in the association reaction. Although the ghosts may have retained their full complement of protein and lipid slight alterations in the configuration of the membrane may have occurred, disorganizing the specific binding sites normally involved in the reaction between transferrin and the cell. A third possibility is that the association reaction is dependent on a transferrin-specific endocytosis [19, 20] and that haemolysis alters the cells in such a way that this type of endocytosis ceases.

Transferrin and albumin uptake by ghosts may occur by adsorption, by diffusion through the membrane or possibly by endocytosis. Damaged cells in general adsorb more protein than intact cells [21]. It seems likely that at least some of the protein uptake by ghosts occurs by this means. Diffusion through damaged areas of the membrane is suggested by the progressive nature of protein uptake at 37 °C for at least 30 min. Evidence from electron microscope studies suggests that permeability of ghosts to large molecules occurs only at the time of haemolysis [23, 24]. In the present work the ghosts were prepared in the presence of Ca^{2+} and then resealed

at 37 °C which has been shown to effectively restore impermeability to cations [25, 26]. However, the possibility of slow permeation by transferrin and albumin cannot be excluded. The observation that labelled proteins incorporated into ghosts at the time of haemolysis were released during subsequent incubation suggests that diffusion through the ghost membranes may have occurred, although it is possible that the protein which did leave the ghosts was that adsorbed to the membrane while protein within the ghosts was not released. The third explanation considered, that of endocytosis, has been shown to occur in erythrocyte ghosts in the presence of ATP [22], but it would not be expected in the absence of added ATP. The effect of ATP on protein uptake which was found in the present experiments could have been due to endocytosis, but if so, it was not specific for transferrin, unlike protein uptake by intact reticulocytes.

The pattern of release of labelled transferrin and albumin from ghosts was similar whether the proteins were incorporated in the ghosts at the time of haemolysis or after the ghosts had been resealed and washed. However, when the labelled proteins were taken up by intact cells from which ghosts were later prepared the release of transferrin was far less than that found with the other methods of uptake while albumin release was similar (Fig. 4). This supports the concept that transferrin and albumin are taken up and held in different manners by intact reticulocytes but that the mechanism of their binding and uptake by ghosts is similar. That is, the characteristics of the specific transferrin uptake process are lost when reticulocytes are haemolysed. One possible explanation for the small degree of release of the transferrin taken up by intact cells is that the protein is bound within the outer cell membrane, possibly within endocytotic vesicles and that the process of exocytosis by which transferrin may be released from intact reticulocytes [20] does not occur after cell integrity is disturbed by osmotic haemolysis.

The effect of pH on transferrin uptake is of interest. It has been shown that incubation of human erythrocyte ghosts at pH 5.5 produces aggregation of the particles observed in the membrane by electronmicroscopy [27]. It is not known whether a similar change occurs in the membrane of intact cells incubated at this pH, but if so, it may be related to the loss of capacity to take up transferrin. The increased uptake of both transferrin and albumin by ghosts pre-incubated at pH 5.5 indicates that the effect of any change produced is not specific for transferrin and is quite different from that which occurs with intact cells. It is possible that the effects of pH and calcium on the uptake of protein by ghosts were dependent on a reduction in the net negative charge on the membranes so that more protein could be adsorbed as a result of electrostatic interactions.

Because hypotonic haemolysis of reticulocytes results in a loss of the specific reaction with transferrin the use of ghosts for the identification and isolation of transferrin receptors is unlikely to be successful.

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