Amines as Inhibitors of Iron Transport in Rabbit Reticulocytes*

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Jonathan Glass$ and Marco Tulio Nunez§

From the Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of the Beth Israel Hospital, Department of Medicine, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02115

The effect of the known inhibitors of iron uptake, n-butylamine and NH₄Cl, was examined at the molecular level to more precisely define the mechanisms by which these lysosomotropic agents block iron uptake by rabbit reticulocytes. Utilizing a rapid pulse-chase technique to follow the handling of a cohort of ⁵⁹Fe,¹²⁵I-transferrin bound to rabbit reticulocytes, both amines were observed to have no effect on the cell-mediated release of ⁵⁹Fe from internalized transferrin. The results indicated, however, that both agents acted to 1) retard the internalization of transferrin bound to transferrin receptors on the plasma membrane of reticulocytes, 2) retard the externalization of internalized transferrin, and 3) block the transport into the cytosol of iron released from transferrin.

The initial step in the delivery of iron to reticulocytes is the binding of the iron transport protein transferrin to specific receptors on the reticulocyte plasma membrane. We have recently described a rapid pulse-chase technique to follow the fate of a single cohort of transferrin bound to its receptor (1). By use of the cohort analysis, it is possible to establish that iron is released from transferrin shortly after transferrin binding. After a dwell period on the cell, apotransferrin is slowly released from the cell, while iron released from transferrin passes through the plasma membrane, cytosol, and mitochondria with the kinetic behavior of an intermediate.

The dwell period of transferrin on the cell is associated with the multiple recent observations demonstrating internalization of the transferrin-transferrin receptor complex by coated vesicles (2–16). The cell compartment into which transferrin is internalized has been characterized as a peripheral (10, 17), acidic (6, 16), nonlysosomal (10, 18) vesicle. These various studies (2–16) demonstrate that transferrin is routed to endosomes and then, in nonhematopoietic cells, to the transreticular portion of the Golgi apparatus (15) or, in hematopoietic cells, to multivesicular organelles (4) prior to exocytosis. During this intracellular journey, transferrin is confined within an acidified vesicle (6, 18). Hence, iron released from the transferrin will have to cross the vesicle membrane to gain access to the cytosol.

Since the various steps in the multistep process of iron delivery to reticulocytes can be distinguished and kinetically defined, it is possible to examine with more precision the step(s) at which inhibitors of iron uptake may act. In the present study, we have examined the action of two amines, n-butylamine and NH₄Cl, both well-described inhibitors of iron uptake (5, 19–21). Primary amines are hypothesized to act by inhibiting acidification of intracellular vesicles, consequently inhibiting acid-induced release of iron from transferrin and prolonging occupancy of the transferrin receptor with diferric transferrin (4, 5, 9, 13, 21). In these studies, we have used the model derived by cohort analysis to test the hypothesis for the action of these amines. The observed effects indicate, however, that the action of these amines at concentrations which completely inhibit iron uptake is to retard the cell-mediated internalization and externalization of transferrin and to block the transport of iron across the plasma membrane.

MATERIALS AND METHODS

Rabbit reticulocytes and rabbit ⁵⁹Fe,¹²⁵I-transferrin were prepared, and cohort analysis including cell lysis, preparation of stroma and cytosol, and determination of fractional saturation of transferrin was performed as previously described (1, 22). Stock solutions of 60 mM NH₄Cl and 30 mM n-butylamine were prepared in phosphate-buffered saline with pH and osmolality adjusted to 7.4 and 300 mosM, respectively.

**Pronase Digestion of Bound ¹²⁵I-Transferrin and of the Transferrin Receptor—Resistance of bound transferrin to Pronase digestion was assayed by incubating 1 volume of reticulocytes with an equal volume of 1% Pronase (Calbiochem-Behring) in PBS (0.2 g of KCl/l, 0.2 g of KH₂PO₄/l, 8 g of NaCl/l, 2.16 g of Na₂HPO₄/l × H₂O) for 15 min at 4 °C. The reaction was stopped by addition of 4 volumes of fetal calf serum, and the cells were washed. Residual cell-associated radioactivity was determined if reticulocytes had previously been incubated with ⁵⁹Fe,¹²⁵I-transferrin. If reticulocytes had previously been incubated with nonradioactive transferrin, transferrin-binding sites were quantitated following Pronase digestion by determining the amount of transferrin bound at concentrations from 0.05 to 2 µM ⁵⁹Fe,¹²⁵I-transferrin with subsequent Scatchard analysis as previously described (23).**

**Binding and Internalization of ⁵⁹Fe,¹²⁵I-Transferrin to Rabbit Reticulocytes—**In this assay, binding of transferrin to reticulocytes is defined as energy-independent binding which occurs at 4 °C, whereas internalization is defined as the energy-dependent association of transferrin with reticulocytes. The rate of binding was assayed by addition of ⁵⁹Fe,¹²⁵I-transferrin to a final concentration of 2 µM to 50 µl of reticulocytes previously preincubated at 37 °C with n-butylamine, NH₄Cl, or PBS and then chilled to 4 °C. Aliquots of 20 µl were removed at varying times, and cells were separated from unbound transferrin by centrifugation through dibutyl phthalate (6). The rate of internalization of ⁵⁹Fe,¹²⁵I-transferrin was assayed in the presence or absence of n-butylamine or NH₄Cl using 200 µl of reticulocytes preincubated at 37 °C for 2 min with either agent or...
PBS alone. The incubations were then made 2 μM in $^{59}$Fe,$^{125}$I-transferrin. Aliquots were removed at varying times, added to 500 μl of iced rabbit plasma, and allowed to sit at 4°C for 60 min prior to washing. In preliminary experiments, these conditions were sufficient to completely exchange greater than 90% of bound, but not internalized, transferrin. The rates of internalization were calculated as the percentage of full saturation of reticulocytes with transferrin.

Binding of $^{59}$Fe,$^{125}$I-Transferrin Receptors in a Solubilized Binding Assay—A soluble binding assay similar to that previously described (24) was used to assess the effect of n-butylamine and NH$_4$Cl on binding of transferrin to the transferrin receptor. One volume of plasma membranes prepared by hypotonic lysis (22) from amine-treated or control reticulocytes was solubilized at 4°C for 5 min with 0.5% Triton X-100 and centrifuged at 25,000 × g for 15 min. Twenty μl of solubilized membrane in the presence or absence of 10 mM n-butylamine or 20 mM NH$_4$Cl was incubated at 37°C for 30 min in a final volume of 40 μl. The samples were iced, a 1.6-μg aliquot of $^{59}$Fe,$^{125}$I-transferrin was added, and incubation was continued for 5 min. Transferrin-receptor complexes were stabilized by decreasing the pH by addition of 150 μl of 0.1 M sodium citrate, pH 5.0 (28). Twenty μg of carrier rabbit IgG was added, and the transferrin-receptor complex was precipitated with an equal volume of 18% polyethylene glycol in 0.1 M sodium citrate, pH 5.0. After 20 min at 4°C, precipitates were collected by centrifugation and washed once with 9% polyethylene glycol in sodium citrate.

RESULTS

Cohort Analysis of Bound $^{59}$Fe,$^{125}$I-Transferrin Effect of NH$_4$Cl and n-Butylamine—In preliminary experiments, $^{59}$Fe uptake ceased completely within 1–2 min of adding of 20 mM NH$_4$Cl or 10 mM n-butylamine and recovered to the rate in untreated cells within 1–2 min of washing the cells free of amines. These findings corroborate the earlier studies of Morgan (19) who found complete inhibition at similar concentrations. Hence, in subsequent experiments using cohort analysis, reticulocytes were preincubated for 2 min with the amines and the amines were included in the chase solution used in the cohort analysis. Fig. 1 illustrates the fate on subsequent chase of $^{59}$Fe,$^{125}$I-transferrin bound to rabbit reticulocytes during a 10-s pulse. As in our initial studies (1), $^{125}$I-transferrin bound to control reticulocytes had a dwell time of about 1 min prior to the commencement of release; release then preceded with a half-time of about 3 min. In striking contrast is the marked stability of bound $^{125}$I-transferrin on n-butylamine- and NH$_4$Cl-treated cells. With butylamine, significant release of $^{125}$I-transferrin was seen only after 10 min of chase, whereas at least 15 min were required with NH$_4$Cl (data not shown). The marked inhibition of transferrin release occurred even if the amines were added only during the chase.

The decreased rate at which transferrin is chased from the treated cells, observed after long incubations of cells with inhibitors, has been suggested to result from a block of release of iron from transferrin (3, 9, 12, 21). As dipher ferric transferrin has a higher affinity for the transferrin receptor than apo-transferrin, the dipher ferric transferrin would remain bound to the cell. However, as seen in Fig. 2, preincubation with either 10 mM n-butylamine or 20 mM NH$_4$Cl did not impede the release of $^{59}$Fe from transferrin. In all three groups, by the end of the 10-s pulse, about 30% of the $^{59}$Fe was already released from transferrin. Release of iron continued at an approximately linear rate so that after 60 s of chase, about 60–70% of the iron had been removed from the bound transferrin.

To investigate the fate of the $^{59}$Fe released from the bound cohort of transferrin, the cells were lysed and $^{59}$Fe radioactivity was measured in the cytosol. During the chase, the control reticulocytes accumulated $^{59}$Fe in the cytosol at a rate of about $2.1 \times 10^9$ molecules of $^{59}$Fe/cell/min (mean of three experiments), whereas no significant accumulation was seen in the cytosol of treated cells.

Amines Alter the Amount of Bound Transferrin Sensitive to Pronase—The inhibition of release of apotransferrin could result from transferrin being trapped in a compartment from which it could not exchange with the external milieu. To test this hypothesis, the sensitivity of bound transferrin to Pronase digestion was examined. After the various chase times, reticulocytes with bound $^{125}$I-transferrin were incubated with Pronase at 4°C and washed, and the residual radioactivity was determined (Fig. 3). At the end of the 10-s pulse, about 50% of the bound $^{125}$I-transferrin was sensitive to Pronase. This decreased to only 20% sensitive to Pronase by 5 min of chase. The per cent protection was the same for butylamine- and NH$_4$Cl-treated cells. Considering the amount of transferrin chased, 80% of transferrin resistant to Pronase digestion
at 5 min in the control cells represents about 15% of the originally bound transferrin inaccessible to Pronase. In the treated cells, as an insignificant amount of transferrin was released by 5 min, 80% protection implies that about 80% of the originally bound transferrin was inaccessible to the Pronase in the external medium.

Butylamine Does Not Affect Transferrin Receptor Sensitivity to Pronase—The increased extent of protection of bound transferrin could result from the amines causing compartmentalization of the transferrin receptor, in which case the number of transferrin receptor/cell resistant to Pronase digestion would be greater in the treated cells. To determine receptor sensitivity to Pronase, reticulocytes were incubated with nonradioactive transferrin in the presence or absence of butylamine for 20 min to assure that all receptors were occupied. The cells were digested with Pronase and washed, and the amount of transferrin which could subsequently be bound per cell was determined at 59Fe,125I-transferrin concentrations from 0.05 to 2.0, μM. By Scatchard analysis of bound transferrin, the nontreated, nondigested cells had about 1.4 × 10^6 receptors, whereas after Pronase digestion, both the amine-treated and -nontreated cells had 6.5 × 10^4 receptors (mean of three experiments). The affinity constant of transferrin for its receptor of 1.7 × 10^1 M⁻¹ in the control cells was unaffected by Pronase digestion or exposure to the amine. These studies suggest that n-butylamine does not affect the distribution of transferrin receptors and that under equilibrium conditions, about half the receptors are oriented to be accessible to Pronase in the external milieu. Similar findings for untreated reticulocytes have been reported by Egged (29).

Rate of Binding and Internalization of 125I-Transferrin—A consistent finding in the pulse-chase experiments was that after the 10-s pulse, about 67% less 125I-transferrin was bound to the treated cells than was bound to the control cells. The amount of bound transferrin is the sum of two steps—binding to the receptor followed by sufficient "internalization" to confer resistance of bound transferrin to exchange from the cell. The first step was assessed by measuring the rate of transferrin binding at 4°C, a step previously shown to be energy-independent (23). No differences were noted between control and amine-treated cells for the rates of transferrin binding at 4°C. With both control and amine-treated cells, the maximum extent of binding at 4°C was about 50% of the maximum achieved at 37°C and occurred by 15 min of incubation. However, when the rate of internalization of transferrin was measured, differences were observed (Fig. 4). In the control cells, the time to half-maximal internalization was 126 s, as compared to 227 s with n-butylamine- and NH₄Cl-treated cells. Hence, both amines had an effect on the processing of transferrin after the initial binding to the receptor. Eventually, receptor saturation of about 75% occurred at about 15 min in the presence of amines, compared to about 8 min with control cells.

Effect of Amines on Transferrin Binding in a Soluble Binding Assay—The ability of transferrin to bind to detergent-solubilized plasma membranes or whole cells has been used to characterize the transferrin receptor (28). We have previously used such a system to demonstrate the direct effect on the transferrin receptor of an inhibitor of transferrin binding (24). To determine if the amines could also directly affect the receptor, the soluble binding assay was performed using both plasma membranes from amine-treated cells and control cells in the presence and absence of amines. No significant differences in binding were seen. Under the conditions of this assay, about 14.5% binding of added transferrin occurred with control membranes, 13.8% with membranes from n-butylamine-treated reticulocytes, and 14.0% with control membranes incubated with 10 mM n-butylamine. If, after binding of 59Fe,125I-transferrin, a 100-fold excess of unlabeled transferrin was added, the labeled transferrin fully exchanged off the receptor with no differences noted in the rate of exchange in the three groups.

DISCUSSION

Amines have been used previously in reticulocytes (19-21) and cell lines (2, 3, 5, 9, 12-14) to block iron transport. In the studies reported here using a technique recently devised by us (1), we have localized more precisely the points of action of these agents. In particular, our studies demonstrate that two inhibitors of iron uptake, n-butylamine and NH₄Cl, used by us and others (19-21) at concentrations which completely inhibit 59Fe uptake act to retard internalization of transferrin bound to the reticulocyte transferrin receptor, to impede release of transferrin from the receptor, and to block the
transport of iron released from transferrin into the cytosol of the reticulocyte.

After binding of $^{59}$Fe,$^{125}$I-transferrin to reticulocytes, the $^{59}$Fe and the $^{125}$I-transferrin rapidly diverge. The $^{59}$Fe is quickly removed from the transferrin, crosses the plasma membrane, and enters the cytosol, whereas the $^{125}$I-transferrin, after a lag period, starts to externalize in a process that takes an additional 3–5 min to complete (Fig. 1 and Ref. 1). At the concentrations of $n$-butylamine and NH$_4$Cl used, neither agent affected the rapid release of iron from transferrin.

It is important to note that our findings are based on direct determination of the iron saturation of cell-bound transferrin, whereas the conclusions of other studies are drawn from the observation that neither $^{59}$Fe nor $^{125}$I radioactivity is released from amine-treated cells (12, 21). In the K562 cell line, after prolonged incubation, NH$_4$Cl does block $^{59}$Fe release from transferrin (13). Whether the effect with K562 cells reflects differences in mechanisms of iron transport between reticulocytes and the K562 cell line or merely the marked alteration of cell structure which occurs after prolonged incubation with NH$_4$Cl (19) has to be resolved. In any event, in reticulocytes, a major effect of $n$-butylamine and NH$_4$Cl on iron transport is to block transport of released iron in the cytosol. Lysosomotropic agents are purported to block iron transport by increasing the pH of endocytic vesicles (2, 3, 8, 11, 12, 14, 19–23). As iron is released from transferrin in the presence of these agents, acidification may be important, not for release of iron from transferrin, but for the translocation of iron through the vesicle membrane subsequent to its release. By alkalinizing an acidic compartment into which iron is released, the amines would raise the hydroxyl ion concentration in such a compartment. The hydroxyl ions could then compete for iron with the physiologic membrane iron binders by forming insoluble complexes of Fe(OH)$_4$. The present studies with amines are in some aspects similar to our recent studies examining the effect on iron uptake in reticulocytes of the ionophores monensin and nigerisin, which exchange protons for monovalent cations (17). At low concentrations, the ionophores also inhibit cellular iron uptake without an inhibition of iron release from transferrin, suggesting that with a slight rise in pH of the compartment into which the transferrin-transferrin receptor complex is internalized, binding of released iron to its membrane carrier is inhibited. As the effect of the ionophores is dependent on the cation composition of the extracellular medium, the compartment in which iron is released from transferrin and bound to its membrane carrier is a peripheral compartment contiguous to the extracellular medium. An alternative locus for the effect of the amines could be at the reduction of Fe(III) to Fe(II). Iron is released from transferrin as Fe(II) and subsequently reduced in the membrane to Fe(II) where it is available to chelation by lipophilic Fe(II) chelators (25). The possibility that iron is trapped as Fe(III) is supported by the observation that in the presence of amines, dipyridyl will not release $^{59}$Fe from reticulocytes (20).

The amines were also observed to affect the handling of transferrin. As noted previously by Armstrong and Morgan (20), the release from the reticulocyte of transferrin bound in the presence of amines is markedly retarded. However, the persistent binding of transferrin to the cell cannot be the result of the higher affinity of diferric transferrin than apo-transferrin for its receptor as neither $n$-butylamine nor NH$_4$Cl inhibited the formation of apotransferrin. To determine if the inhibition of externalization was the result of increased compartmentalization of transferrin, sensitivity to Pronase of bound transferrin was examined. The initial observations demonstrated that the amines did not increase the per cent of protection although the extent of protection was increased as an insignificant amount of transferrin was chased. This result could have been caused by an amine-induced sequestration of transferrin-transferrin receptor complex into a compartment to which Pronase had no access. If sequestration were occurring, then under equilibrium conditions when receptors were fully occupied with transferrin, more receptors in the amine-treated cells should be hidden from Pronase. In this instance, upon subsequent removal of the amine, the previously treated cells should be able to bind more transferrin than control cells. However, as we observed that Pronase decreased receptor number equally in amine-treated and control cells, the amines must be acting not by sequestering the transferrin-receptor complex, but by retarding transferrin release from the receptor. Parenthetically, Pronase resistance does not necessarily imply internalization into a vesicle, as we could demonstrate that transferrin bound to anti-transferrin antibodies attached to Sepharose beads was 60% protected from Pronase digestion (data not shown). The wider interpretation is that Pronase resistance may reflect masking of Pronase-sensitive sites by protein-protein interactions.

Not previously described is the effect of the amines on the initial handling of transferrin by reticulocytes. The "on-reaction" in reticulocytes (25) is composed of two steps: the reversible binding of transferrin to the receptor and the internalization of bound transferrin. The first step occurs at 4°C and was not affected by either agent, whereas the second energy-requiring step was affected. To see the effect of the amines on the on-reaction, it was necessary to examine the initial rates of internalization. Although these rates were decreased by nearly 2-fold, the receptors could eventually be filled with transferrin. These observations explain the failure of previous studies to detect an appreciable effect on the on-reaction (21). Hence, these amines affect both the initial handling (internalization) and final disposition (externalization) of transferrin by reticulocytes, as well as the transport of released iron. These parameters are minimally affected when iron uptake is inhibited by ionophores (17).

The effects of amines are compatible with the model of iron uptake derived from cohort analysis (1), studies with chelators (27), and use of ionophores which define iron release as occurring in a peripheral compartment (17), as well as the numerous recent studies demonstrating transferrin internalization (2–16). It is difficult to precisely compare the models derived from our system (1, 17, 27) and those of others (2–16) as the systems differ markedly with various of the later studies employing longer incubations, modified transferrins, cell lines (2, 3, 5–16), and nonhomologous transferrin (4). Nonetheless, the amines appear to affect the dwell time of transferrin on the reticulocyte (corresponding to the period of internalization) as well as uptake of released iron (corresponding to the transport of iron from vesicle interior into the cytosol). If the amines act only at one locus, e.g. alkalinizing the intravesicular space, then it can be speculated that transferrin processing and iron transport across the membrane, although kinetically asynchronous, may be coupled processes.

REFERENCES

6. Renswoude, J. V., Brügge, K. R., Harford, J. B., and Klausner,
Amines as Inhibitors of Iron Transport in Rabbit Reticulocytes