Iron Uptake by Human Reticulocytes at Physiologic and Sub-Physiologic Concentrations of Iron Transferrin: The Effect of Interaction With Aluminum Transferrin

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We have studied the interaction, in vitro, between diferric transferrin (FeTr), aluminum transferrin (AlTr), and human reticulocytes harvested from human placental blood. In particular, we aimed to determine the extent to which the kinetics of AlTr and FeTr differed. Using transferrin labeled with either \( ^{59}Fe \) or \( ^{59}Al \), the association of radiotracer with reticulocytes, as a function both of time and of transferrin concentration, was examined. Under the conditions of the experiments, the data are consistent with a mechanism involving at least three processes. Two early processes acting in parallel behave as a high-affinity saturable receptor and a low-affinity non-saturable receptor, neither of which distinguish between AlTr and FeTr. In a subsequent process, AlTr and FeTr exhibit different kinetics. This third process may be saturated by FeTr but not by AlTr. Interpreted in terms of a current conventional view of metallo-transferrin uptake, we conjecture that the early parallel processes involve cell surface phenomena including classical transferrin-receptor binding, and that the subsequent process represents events, possibly intracellular, involved in metallo-transferrin dissociation or further iron transport. The extent to which AlTr influences the interaction of FeTr with reticulocytes offers insight into both the normal physiology of iron uptake and the potential for toxicity by aluminum.

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similarly, using ferric citrate. Radioiron, 0.5 mCi as \(^{59}\text{FeC1}_3\) (Amersham Int, Amersham, UK) in 0.1 N HCl was added to an equal volume (0.5 mL) of a solution of 1 mmol/L Na citrate, 100 mmol/L NaHCO\(_3\) shaken, and then added with approximately 20 mg apotransferrin in the Tris buffer and left 4 hours at 4°C. A 2.5-fold molar excess of Fe citrate was then added and this solution left overnight and washed and concentrated as already described. Iodinated metallo-transferrin solutions were prepared using the lactoperoxidase procedure.\(^{22}\) The entire solution to be used was iodinated using \(^{125}\text{I} (Amersham Int) with gentle oxidation, rather than using a small amount of potentially denatured \(^{125}\text{I}-\text{transferrin} as a tracer in a solution of native metallotransferrin. Samples of the FeTr and AlTr were analyzed for Fe, Al, and transferrin using, respectively, the ferrizine dye binding method (Roche Diagnostics, Basel, Switzerland), electrothermal atomic absorption spectroscopy,\(^{23}\) and a turbidimetric assay.\(^{24}\)

**Reticulocyte harvest.** Informed consent was obtained in writing from pregnant women nearing delivery. The placental blood was collected into heparinised tubes and stored at 4°C until used experimentally (not more than 6 hours later). Samples with clots were not used. The whole blood was centrifuged at 150g for 20 minutes in a Centra 3 machine (IEL Damon, Needham Heights, MA) and approximately 4 mL of red blood cells (RBC) aspirated from just beneath the buffy coat. The RBC were suspended in 50 mL warm saline and centrifuged at 150g for 30 minutes at 4°C. The supernatant was removed and the slurry portioned out into 400-µL centrifuge tubes (Beckman, Palo Alto, CA) as needed. The slurry was then suspended in an equal volume of minimum essential medium (Sigma) at 37°C. The RBC were centrifuged as before, the supernatant removed, and the slurry portioned out into 300-µL centrifuge tubes (Beckman, Palo Alto, CA) as needed. The slurry was kept carefully mixed and the first 1 mm of the disposable tip of the pipette was removed to prevent its becoming blocked by RBC. A sample of known volume of slurry was analyzed in an automatic counter (Cash:Coulter, Hialeah, FL) for hematocrit and a thin film of slurry was stained with New Methylene Blue. Reticulocytes were counted from approximately 2 × 10\(^5\) cells.

**Reticulocyte incubation.** Incubation was performed in prewarmed microfuge tubes placed in a heating block in a shaking water bath. In pilot studies, a thermocouple (Comark, Littlehampton, UK) was used to ensure that the contents of the tubes were at 37°C. The reagent mixture consisted of 30 µL of human albumin 40 g/L, 100 µL of the transferrin solution, and 100 µL of the competing transferrin solution or the Tris buffer, as appropriate. An air gap separated this mixture from the RBC slurry 100 µL, placed above. At the start of the incubation, the tubes were vigorously shaken. After incubation, the tubes were centrifuged for 20 seconds in a type 152 microfuge (Beckman) and stood in ice. The supernatant was immediately aspirated and replaced with ice-cold saline 150 mmol/L. The cells were resuspended and this washing procedure repeated twice more. After the final centrifugation, the tube tips with the RBC pellet were cut off and counted for radioactivity. Each experiment was performed simultaneously in duplicate.

**Time-course experiments.** Portions (100 µL) of RBC slurry were incubated in \(^{59}\text{FeTr}\) at 5 µmol/L final concentration across a range of times from 0 to 60 minutes. Three such experiments were performed. Similarly, RBC were incubated with \(^{125}\text{I}-\text{FeTr} at 5\) µmol/L and separately with \(^{125}\text{I}-\text{AlTr} at 5\) µmol/L, across a range of times from 0 to 38 minutes (two experiments). The curve of uptake of \(^{125}\text{I}-\text{FeTr} with respect to time was analyzed by dividing the plateau value by the initial gradient to give a weighted harmonic mean time constant for the transferrin cycle.

**Comparison of \(^{59}\text{FeTr uptake at subphysiologic and physiologic concentrations.}** The portions of RBC slurry were incubated for 60 minutes with \(^{59}\text{FeTr} across the concentration ranges 0.2 to 5 µmol/L and 0.2 to 33 µmol/L. Five such experiments were performed for each range. In two of each of these experiments, observations were also made using identical mixtures at 4°C.

**Inhibition of \(^{59}\text{Fe uptake by AlTr.}** Incubations were performed over 60 minutes across the range of \(^{59}\text{FeTr concentrations of 0.2 to 6 µmol/L using 100 µL of Tris buffer but no AlTr, with replicate tubes containing 100 µL of AlTr at the fixed concentration 1.5 µmol/L in each tube (Al:Fe ratio 0.25 in the tube containing the highest \(^{59}\text{FeTr concentration.}** Simultaneous incubations, using the fixed AlTr concentrations (in µmol/L), 3, 4.5, 7.5, or 13.5 were performed, but in any single experiment it was only possible to study the effect of AlTr at three of these concentrations in addition to the incubation without AlTr. Five experiments were performed testing all of the selected Al:Fe ratios. Five experiments at the same ratios were performed using the range of \(^{59}\text{FeTr concentrations 2 to 33 µmol/L.}** After incubation, the RBC were washed and counted as described.

**Competitive binding experiments.** Experiments were performed using short (12 minutes) and long (60 minutes) incubations with a view to distinguishing between immediate processes, such as binding to the cell surface, and those that were ongoing, such as the accumulation of \(^{59}\text{Fe} within the cell.** We therefore examined the uptake of \(^{59}\text{Fe from 1.0 µmol/L \(^{59}\text{FeTr during 60 minutes of incubation in the presence of a range of concentrations of FeTr, and separately of AlTr, up to the concentration 50 µmol/L.}** Three such experiment of this type were performed with different batches of RBC. One similar experiment was repeated using a 5-minute incubation period. Three comparable experiments were also performed using 1.0 µmol/L \(^{59}\text{I}-\text{FeTr during 12 minutes of incubation.}** In all cases, duplicate experiments were performed at 4°C.

**Analysis.** All data points are the mean of duplicate observations. They were tabulated and analysed on a Lotus 1-2-3 spreadsheet (Lotus Development Corp, Cambridge, MA), using an IBM 386PC. The results of rate of \(^{59}\text{Fe uptake in relation to \(^{59}\text{FeTr were analyzed} by both Lineweaver-Burke and Scatchard methods. Hill plots were used for data of \(^{59}\text{Fe uptake from \(^{59}\text{FeTr in the concentration range 0.25 to 2.5 µmol/L.}** In experiments incorporating either FeTr or AlTr as a competitor to \(^{59}\text{FeTr, data were analyzed} by Lineweaver-Burke plots and by the method of Dixon and Webb.\(^{25}\) The \(K_K\) for AlTr in the \(^{59}\text{FeTr-reticulocyte system was calculated from secondary plots of the Lineweaver-Burke data, in which the gradient of the primary plot was related to the [AlTr]. The intercept on the ordinate was the \(K_K. Results in the text have been expressed as median and range for nonparametric data. Otherwise, mean and SD has been used.

**RESULTS**

**Metallo-transferrin preparation.** Spectroscopic titration of apotransferrin with \(\text{AIK(SO}_4\text{)}_2\) showed 1:2 molar binding and analysis of both AlTr and FeTr routinely showed that the protein was 75% to 95% saturated. The specific activity of \(^{59}\text{FeTr was 1.2 × 10^{4giec}}} pmol/mole.** For the \(^{125}\text{I}-\text{FeTr} and \(^{125}\text{I}-\text{AlTr}, the values were, respectively, 5 × 10^{15} and 6 × 10^{15} pmol/mole protein.

**Reticulocyte harvest.** The reticulocyte count in the slurry obtained from the simple centrifugation procedure was 5.6% ± 3.0% of total RBC. No white cells were obtained.

**Iron uptake in absence of AlTr.** The RBC pellets accumulated \(^{59}\text{Fe in direct proportion to time during incubation at 37°C with \(^{59}\text{FeTr. However, there was no progressive uptake at 4°C. As a function of time, the interaction of \(^{125}\text{I}-\text{FeTr 5 µmol/L with the RBC approached its maximum**
exponentially with a weighted harmonic mean time constant of 4 minutes.

Uptake of $^{59}$Fe over 60 minutes plotted against [FeTr] gave a relationship that was initially hyperbolic but was followed by a steady increase observed beyond an [FeTr] of approximately 5 μmol/L. (Fig 1, inset). Scatchard plots indicated two phases for uptake, with the high-affinity phase predominating at low [FeTr] and a low-affinity phase clearly operating at [FeTr] greater than 5 μmol/L. (Fig 1). The mean value of $K_b$ for the high-affinity binding was 3.00 (±1.67) μmol/L determined by Scatchard analysis and 3.21(±2.00) μmol/L by the Lineweaver-Burke method. Taking 4 minutes as the value for the recycling time for $^{125}$I-FeTr, the mean receptor density calculated for the high-affinity receptor ranged from 0.3 to 16 x 10^4 (median 1.1 x 10^4) sites per cell from the Scatchard analysis. Using these data, Hill plots gave gradients of value close to unity (0.90 ± 0.03). We have not attempted to derive a low-affinity $K_b$ value or the theoretical number of these binding sites per cell.

**Time course of $^{125}$I-FeTr uptake.** When RBC were incubated with 5 μmol/L $^{125}$I-AlTr for up to 38 minutes, the accumulation was 1.8-fold that of $^{125}$I-FeTr under the same conditions and was continuing to increase, whereas the $^{125}$I-FeTr uptake had reached a plateau (Fig 2).

**Competition between labeled FeTr and unlabeled FeTr or AlTr.** In the competitive binding experiments, with 60 minutes of incubation, addition of unlabeled FeTr produced a progressive reduction in $^{59}$Fe uptake by the cells to less than 4% of the maximal value (Fig 3). A plot of the reciprocal of $^{59}$Fe uptake against the unlabeled FeTr concentration (Dixon method), for concentrations less than 5 μmol/L, gave a linear relationship (Fig 4). At higher [FeTr], the relationship remained linear but the slope increased by a factor of 20%.

When AlTr was used in place of the FeTr in the experiment described above, the effect of competitive binding at concentrations less than 5 μmol/L was identical to that seen with the FeTr. However, at [AlTr] greater than 5 μmol/L, there was no further inhibition of $^{59}$Fe uptake at all, in contrast to the effect with FeTr (Figs 3 and 4).

A different picture emerged from the results of the experiments designed to highlight ligand interaction with the receptor, rather than overall $^{59}$Fe accumulation within the cell. Thus, in the experiments performed at 4°C, uptake of $^{59}$Fe (in the absence of FeTr or AlTr) was 8% (6% to 11%) of the corresponding value at 37°C but was only reduced to 16% (14% to 20%) (compared with 4% at 37°C) and 25% (24% to 26%) of this maximal value by the addition of excess FeTr or AlTr, respectively. In the 5-minute 37°C incubation experiment, the FeTr only reduced uptake of $^{59}$Fe to 30% and the AlTr to 49% of the maximal value. This difference was even more obvious when we used $^{125}$I-FeTr as the tracer, with FeTr in competition during 12 minutes of incubation at 37°C. At total [FeTr] less than 2.5 μmol/L, there was again displacement
Fig 3. The competitive inhibition of 59Fe uptake after 60 minutes of incubation using unlabeled FeTr (---) or AlTr (----).

Fig 4. Dixon plot of data in Fig 3. The reciprocal of uptake is plotted against the concentration of the competing ligand.

Fig 5. The effect of increasing concentrations of FeTr or AlTr on uptake of 125I-FeTr over 12 minutes. Contrast with Fig 2. Symbols are as before.

of the 125I-FeTr from the cells as would be predicted from simple competitive binding considerations. However, at total [FeTr] greater than 5 μmol/L, a maximal degree of displacement of 58% (51% to 64%) of the 125I-FeTr was observed. At these higher concentrations of FeTr, the 125I-FeTr continued to interact with cells in an apparently nonsaturable way. The effect of unlabeled AlTr in the same system gave results almost identical to those observed for FeTr (Fig 5).

When we studied the 59Fe uptake from 59FeTr (concentration range 0.2 to 6 μmol/L) in the presence of a fixed [AlTr], Lineweaver-Burke analysis showed lines focusing on the ordinate, which indicated competitive inhibition (Fig 6). The experiments using higher concentrations (2 to 22 μmol/L) of 59FeTr and correspondingly higher total metallo-transferrin concentrations resulted in Lineweaver-Burke plots indicating partial competitive inhibition. When low [AlTr] were used relative to the concentration of 59FeTr, which maintained the total metallo-transferrin concentration in the solution less than 5 μmol/L, the estimated K was 0.69 (±0.25) μmol/L, which is significantly lower than the K value for FeTr and the high-affinity receptor (P < .01).

**DISCUSSION**

These studies were designed to examine the interaction between AlTr and FeTr in human reticulocytes. The cells used by us appeared to be relatively mature because normoblasts in the film were uncommon. Nonetheless, when low concentrations of FeTr were used, our experi-
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In the presence of AITr, the AlTr concentrations in successively steeper lines were: 0, 1.5, 3, and 4.5 μmol/L. The maximum 59FeTr concentration was 6 μmol/L.

Fig 6. Lineweaver-Burke plot of 59Fe uptake and FeTr concentration in the presence of AITr. The AITr concentrations in successively steeper lines were: 0, 1.5, 3, and 4.5 μmol/L. The maximum 59FeTr concentration was 6 μmol/L.

In the displacement experiments in which increasing concentrations of unlabeled FeTr were used in competition with 59FeTr, with initial concentrations of 1.0 μmol/L, during 60 minutes of incubation, there was ultimately nearly complete inhibition of 59Fe uptake as would be anticipated and less than 2% was accounted for by nonspecific binding (NSB) (Fig 3). When plotted by the method of Dixon, the data were slightly different at [FeTr] less than 5 μmol/L, compared with those at [FeTr] greater than 5 μmol/L. At the low concentrations, the relationship of the reciprocal of rate of uptake on [FeTr] was linear, with a finite intercept on the ordinate, dependent on the 59FeTr concentration. At higher values of [FeTr], the relationship was also linear but the gradient was 20% steeper. The linearity in each case is consistent with progressive saturation of a step in each of two separate transport mechanisms operating at the low and high concentrations.

Because two molecules of FeTr bind to each high-affinity receptor, some form of cooperativity might have been anticipated. However, plots using the Hill transformation of the low concentration data gave relationships with gradients very nearly unity, implying that there was no cooperativity among high-affinity receptors. The Hill analysis could not be applied to the high-capacity system; however, because the gradient of the relation between the reciprocal of 59Fe uptake and [FeTr] in the Dixon plot of the competitive binding data was steeper at high concentrations than at low, there is a strong suggestion that some form of impedance to accumulation of 59Fe was present during uptake by the low-affinity, high-capacity system (Fig 4).

When 60 minutes of incubation at 37°C was performed with AlTr instead of FeTr competing with 59FeTr, a distinct difference emerged between the behavior of the two forms of metallo-transferrin: the AlTr was unable to prevent 59Fe uptake with approximately 25% of maximal 59Fe uptake continuing to take place. The Dixon analysis showed that at [AlTr] less than 5 μmol/L, the AlTr and FeTr displaced 59Fe in a similar way, but at [AlTr] greater than 5 μmol/L there was no competition whatever for the low-affinity process (Fig 4). Lineweaver-Burke analysis showed that the AlTr reduced 59Fe accumulation by competitive inhibition. As the [AlTr] was increased, and with it, its interaction with the high-capacity system, the inhibitory effect became relatively less.

Incubations for 12 minutes with 125I-FeTr 1.0 μmol/L in the presence of increasing concentrations of either FeTr or AlTr showed that at low total concentrations, where uptake by the high-affinity receptor was predominant, the FeTr displaced the 125I-FeTr as expected. The receptors would have begun to become saturated at total [FeTr] in the range of 2 to 3 μmol/L (Fig 1). Thus, the rate of Fe uptake would have increased by only 10% despite the fact that the total [FeTr] was doubled, from 1 μmol/L to 2 μmol/L, by addition of unlabeled FeTr to the 125I-FeTr. This means that the observable rate of uptake of FeTr (only half of which would have been detected as 125I-FeTr) should have decreased to 55% of the initial rate. Any difference from this
value would have been accounted for by true NSB. In the example shown (Fig 5) the counts decreased to 56%, which has the important corollary that true NSB was of very small magnitude. This being the case, that rate of uptake of $^{125}$I-FeTr that was independent of total [FeTr] in the displacement experiment and would classically be interpreted as NSB must be explained in another way, namely, the interaction of the FeTr with the low-affinity, high-capacity receptor. A further difference emerged in this experiment when compared with that using the 60-minute 37°C incubation, where the $^{125}$I-FeTr was not saturated, addition of further metallo-transferrin, either in the form FeTr or AlTr, did not lead to further displacement of the $^{125}$I-FeTr from the RBC; in either case, 30% to 40% of cell-associated transferrin remained in the labeled form. When competitive inhibition experiments with FeTr were performed at 4°C, or for a short incubation period at 37°C, uptake was affected in a similar way to that observed with AlTr. Thus, experiments testing the interaction between FeTr and the receptors, namely, those using iodinated protein, a metabolically inactive state, or a short incubation period, gave a different type of result from incubations measuring accumulation of $^{59}$Fe within the cell. These contrasting findings suggest that the observed competition between labeled and unlabeled FeTr at the higher concentration was not occurring at a binding site, but at a later stage that could be concerned with either release of iron or its subsequent transport into the cell. A hinderance to uptake of $^{59}$Fe after the stage of transferrin-membrane association would provide the most logical explanation for the increase in gradient of the Dixon plot already noted when high concentrations of FeTr were present.

According to the model that is commonly described, following surface binding, the ferric or diferric transferrin is internalized with the receptor, perhaps following a dephosphorylation step and the metal stripped off in an acidic compartment. Both the metallo-transferrin and the subsequently formed apotransferrin continue to bind avidly at a low pH and the latter, therefore, recirculates with the receptor to dissociate at the surface. We found that the $K_v$ for AlTr was slightly lower than the $K_v$ for FeTr. The simplest explanation for this is that AlTr disengaged from the receptor less readily than did FeTr. The cell may therefore be unable to separate aluminum from AlTr, which at first sight is paradoxical because the $K_v$ is much less for aluminum and transferrin than for ferric iron. However, an important difference between AlTr and FeTr is that the latter can be reduced to ferrous transferrin, where the $K_v$ is much lower than that for AlTr (that reduction of ferric iron is a necessary step for separation from transferrin has not been conclusively demonstrated, but must be considered a reasonable hypothesis).

In our time-course experiments comparing uptake of $^{125}$I-FeTr and $^{125}$I-AlTr, the association of the latter with the RBC increased more rapidly than did the former. Moreover, while the $^{125}$I-FeTr taken up by RBC reached a plateau, in the case of $^{125}$I-AlTr there was a persistent upward drift in the $^{125}$I-AlTr associated with the RBC. This result either means that receptor numbers were increasing or that the labeled metalloprotein, perhaps with the receptor, had remained within the cell.

The data presented here emphasize the importance of the diffusion-dependent uptake of metallo-transferrin that in the mid-physiologic range may account for an extra 20% over and above uptake mediated by the classical receptor pathway (Fig 1). The mechanism of this diffusion-dependent process remains an enigma but it must presumably take place close to the high-affinity receptors, so that the same conditions apply for extraction and transport of internalized iron. However, in contrast to the high-affinity pathway where saturation of the receptors is the limiting condition, the high-capacity system is limited at a later point that we tentatively suggest is at the intramembranous iron carrier, the step that follows iron release from transferrin. We further suggest that AlTr and FeTr interact with the cell surface in a closely similar way, but on internalization much of the AlTr remains intact. Subsequent intracellular release of the aluminum may require degradation of the metalloprotein.

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