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The effect of lead on iron uptake from transferrin in human erythroleukemia (K562) cells

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The effect of lead on cellular iron metabolism has been investigated using human erythroleukemia (K562) cells. When the cells were cultured with 100 μM Pb^{2+} for 48 h, the rate of cellular iron uptake from transferrin decreased to 46% of that in untreated cells. Scatchard analysis of the binding data revealed that this reduction was the result of a decrease in the number of transferrin receptors rather than an alteration in ligand–receptor affinity. The results of immunoprecipitation of transferrin receptors on the cell surface also confirmed the decreased expression of transferrin receptors by lead-treated cells. The down-regulation of transferrin receptors by treatment with lead did not result from a decrease in the total amount of the receptor, as determined by immunoblotting. Moreover, the biosynthesis of the receptor was unaffected by lead treatment. Thus, the down-regulation of surface transferrin receptors in lead-treated cells might be due to a redistribution of receptors rather than an actual loss of receptors from the cell. Using kinetic analysis, it was shown that redistribution of the receptor did not result from the alteration in the rates of transferrin receptor recycling. A comparison of the amounts of transferrin receptor on the cell surface and in the cycling pool revealed that the sequestration of the receptor from normal flow through the cycle might cause down-regulation of the surface receptor.

Keywords: iron, lead, transferrin, transferrin receptor

Introduction

Lead is a toxic metal, widely distributed in the environment, that causes a variety of health disorders including anemia. Studies in experimental models and in severe acute lead poisoning in man indicate that lead can inhibit the biosynthesis of heme (Albahary 1972), which may result in anemia. In contrast to the extensive studies on the inhibitory action of lead on enzymes involved in several stages of heme synthesis (Campbell *et al.* 1977, Bottomley & Muller-Eberhard 1988), the possibility that lead can cause a functional impairment in the utilization of iron has not been studied well.

Physiologically, iron delivery to cells is mediated by the iron carrier transferrin, which delivers iron to cells after its binding to specific cell surface transferrin receptors (Bomford & Munro 1985). The cellular iron uptake process involves endocytosis of the diferric transferrin–receptor complex, acidification of the endosome which results in the release of iron and the subsequent return of the transferrin–receptor complex to the cell surface (Bomford & Munro 1985). Thus, cellular iron uptake could be modulated by altering the function of the transferrin receptor. There exists multiple mechanisms by which receptor function might be changed. Receptor function may be directly modulated by a change in the affinity of the receptor for the ligand. The actual number of receptors can be altered by changes in the rates of biosynthesis and/or degradation. Alternatively, a shift in the location of the receptors within the cell may render them more or less available without changing the total cellular content of receptor molecules.

The purpose of the study presented here was to assess the effect of lead on cellular iron uptake from transferrin and to determine which of the mechanisms described above is involved, using human erythroleukemia (K562) cells. We found that K562

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cells cultured with Pb^{2+} exhibited a decreased rate of iron uptake accompanied by the down-regulation of cell surface transferrin receptors.

Materials and methods

Human apotransferrin was purchased from Sigma (St Louis, MO, USA). RPMI 1640 medium and fetal calf serum (FCS) were from Gibco. L-[^{35}S] Methionine (800 Ci/mmol), Na ^{125}I (17 Ci/mg) and $^{59}FeCl_3$ (15 mCi/mg) were obtained from New England Nuclear (Boston, MA, USA). The Durapore filters were from Millipore (Bedford, MA, USA). Transferrin was labeled with ^{125}I or ^{59}Fe as described previously (Taketani *et al.* 1985). Iron-saturated transferrin was used in all experiments.

Cells

Human erythroleukemia (K562) cells were routinely propagated in RPMI 1640 medium in the presence of 10% FCS. Cell density was maintained in the range 1–4 $\times 10^5$ cells ml^{-1}.

Antibodies

Anti-human transferrin receptor polyclonal antibody was raised in rabbits by immunizing them with transferrin receptor purified from placenta (Taketani *et al.* 1987). Ascites containing monoclonal antibody (5E9) to human transferrin receptor (Haynes *et al.* 1981) were prepared as described (Kohno *et al.* 1986).

Iron uptake by K562 cells

K562 cells grown in RPMI 1640 medium containing 10% FCS were washed and suspended in RPMI 1640 medium containing 1% bovine serum albumin and 10 mM HEPES buffer (pH 7.4). The cell suspension (5×10^6 cells) was then incubated with ^{59}Fe-loaded transferrin (500 c.p.m.mg^{-1}) at 37 °C for 30 min. The cells were washed three times with ice-cold phosphate-buffered saline (PBS) and the cell-associated radioactivity was determined with a Packard gamma-counter.

Binding of transferrin to cell surface transferrin receptor

A binding assay using [^{125}I]transferrin was performed as described previously (Taketani *et al.* 1985). K562 cells (1×10^6) were incubated with various concentrations of [^{125}I]transferrin in 100 μl of RPMI 1640 medium containing 10 mM HEPES buffer (pH 7.4) at 4 °C for 90 min. To assess non-specific binding, a 100-fold excess of cold transferrin was added. After the incubation, the cells were washed with ice-cold PBS. The cell pellet was then solubilized as described and the radioactivity was determined with a Packard gamma-counter. The data were analyzed according to Scatchard (1949).

Immunoprecipitation of surface transferrin receptors

K562 cells (1×10^6) cultured with or without Pb^{2+} were incubated at 4 °C for 2 h with 50 μl of ascitic fluid containing the monoclonal antibody (5E9) against human transferrin receptor. After washing with PBS, the cells were lysed with lysis buffer (10 mM Tris–HCl, pH 7.5, containing 2% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) at 4 °C for 20 min. The resulting supernatant was incubated with Protein A–Sepharose at 4 °C for 1 h. The beads were washed six times with the same buffer containing 50 mM EDTA and 0.65 M NaCl. The protein was eluted with Laemmli's sample buffer (Laemmli 1970). The transferrin receptor was resolved by SDS–PAGE on a 7.5% gel. The band was visualized by Coomassie brilliant blue staining.

Labelling of cells

K562 cells (1×10^6) cultured with or without Pb^{2+} were incubated with methionine-free RPMI 1640 medium containing 10% dialysed FCS at 37 °C for 1 h. [^{35}S]Methionine was then added, followed by a further incubation at 37 °C for 4 h. After labeling, the cells were washed with PBS and lysed as described above. The lysate was precleared with normal immunoglobulin (IgG)-coupled Sepharose, followed by incubation with 5E9-coupled Sepharose at 4 °C for 2 h. The beads were washed as described above and the bound protein was analyzed by SDS–PAGE, followed by fluorography.

Immunoblotting

Samples for SDS–PAGE were prepared by boiling K562 cells (1×10^6) in 0.1 ml of Laemmli's sample buffer for 3 min. They were then analyzed on a gel containing 8% acrylamide by the method of Laemmli (1970). Electrical transfer of the proteins to a Durapore filter was as described elsewhere (Taketani *et al.* 1987). The transferred filter was blocked with 3% skimmed milk in Tris-buffered saline and then incubated with anti-transferrin receptor polyclonal antibody. The band containing the receptors which had reacted with the antibody was detected using horse radish peroxidase-conjugated anti-IgG as the second antibody and was quantitated by a Shimadzu CS-900 densitometer.

Endocytosis of transferrin

Cells (5×10^6) were preincubated in RPMI 1640 medium containing 200 μg ml^{-1} [^{125}I]transferrin for 30 min at 4 °C, washed with the same medium containing 200 μg ml^{-1} cold transferrin and then incubated at 37 °C. At various times, aliquots of cells were taken from the culture and added to an equal volume of 0.25 M acetic acid/0.5 M NaCl on ice. After 5 min, a 1/4 volume of 1 M sodium acetate was added. Then the cells were washed twice with ice-cold PBS and cell-associated radioactivity was measured by a gamma-counter.

Dissociation of transferrin from K562 cells

Cells (5×10^6) were incubated with [^{125}I]transferrin as described above for 20 min; they were then cooled by the addition of ice-cold medium, washed free of unbound radioactive ligand at 4 °C using serum-free medium and then resuspended in RPMI medium containing 10% FCS prewarmed to 37 °C. Aliquots containing 10^6 cells were withdrawn from the incubation mixture at various intervals, immediately washed with ice-cold PBS and the radioactivity associated with the cell pellet was determined as described above.

Results

Effect of lead on iron uptake from transferrin

To evaluate the effect of lead on cellular iron metabolism, the iron uptake from transferrin by lead-treated K562 cells was determined and compared with that of untreated cells. A time course of iron uptake from transferrin by both treated and untreated cells is shown in Figure 1. It is clear that the rate of iron uptake by lead-treated cells was lower than that of untreated cells. After 2 days of treatment with 100 μM lead nitrate, the rate of iron uptake was reduced to 40% of the control. This reduction is not attributable to loss of cells since lead treatment did not affect cell viability.

Effect of lead on transferrin receptors on the cell surface

To explore a potential mechanism by which lead treatment might reduce the cellular iron uptake from transferrin, we examined transferrin receptor expression on untreated and lead-treated K562 cells. By immunoprecipitation of transferrin receptors on the cell surface, it was determined that lead-treated cells exhibited markedly fewer transferrin receptors than untreated cells (Figure 2). Thus, lead-treated K562 cells exhibit decreased transferrin expression.

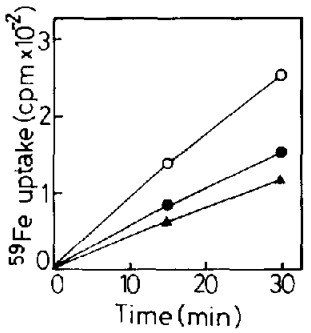

Figure 1. Effect of lead on the rate of iron uptake. K562 cells were cultured with 50 μM (●), 100 μM (▲) or without (○) Pb^{2+} for 48 h. The rate of ^{59}Fe uptake from transferrin was measured as described under Materials and methods.

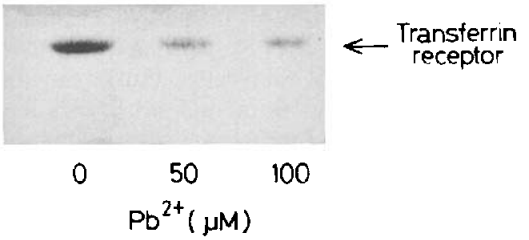

Figure 2. Effect of lead on the amount of cell-surface transferrin receptors. K562 cells were cultured with 50 μM, 100 μM or without Pb^{2+} for 48 h. Transferrin receptors on the cell surface were selectively immunoprecipitated with monoclonal antibody and analyzed by SDS–PAGE. The gel was stained with Coomassie blue.

To quantitate the reduction in transferrin receptor expression more precisely, and to assess the binding affinities of transferrin receptors on lead-treated and untreated K562 cells, we examined transferrin receptors by Scatchard analysis. We incubated lead-treated and untreated K562 cells with various concentrations of [^{125}I]transferrin at 4 °C for 90 min and measured the amount of transferrin bound to the cells. Scatchard analysis of this equilibrium binding data revealed a single class of high affinity receptors on both lead-treated and untreated K562 cells (Figure 3). The number of receptors on untreated K562 cells was 1.1×10^6, whereas lead-treated cells had 4.6×10^5, a reduction of 57%. Lead-treated cells had a similar affinity (K_d = 5.0 nM) to that of untreated cells (K_d = 4.2 nM).

Effect of lead on biosynthesis of transferrin receptors

It has been established that the increase in transferrin receptor expression which is observed after cells

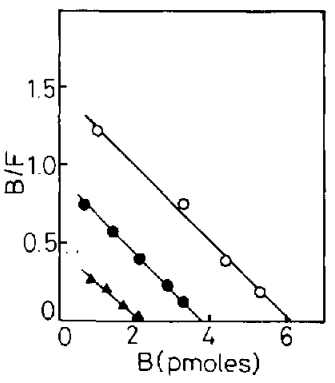

Figure 3. Scatchard analysis of transferrin binding to surface receptors of control and lead-treated cells. K562 cells were cultured with 50 μM (●), 100 μM (▲) or without (○) Pb^{2+} for 48 h. [^{125}I]Transferrin binding to the cells at 4 °C was measured as described under Materials and methods.

are stimulated to proliferate is largely due to *de novo* receptor synthesis (Pausa *et al.* 1984, Taketani *et al.* 1985). In addition, we have previously shown that the decrease in transferrin receptor expression in phorbol ester-treated K562 cells is attributable in part to a reduction in biosynthesis of the receptors (Kohno *et al.* 1986). We therefore attempted to establish whether or not the reduction of transferrin receptor expression by lead treatment was due to a decrease in the receptor biosynthesis. For this purpose, lead-treated and untreated K562 cells were metabolically labeled with [^{35}S]methionine, from which newly synthesized transferrin receptors were immunoprecipitated. As shown in Figure 4, no difference in biosynthesis of the receptors was found between the two types of cells. Thus, the hypo-expression of surface transferrin receptors seen in lead-treated cells might be due to redistribution of the receptor and not to a decreased level of receptor biosynthesis.

Effect of lead on the cellular content of transferrin receptors

Since transferrin receptors continuously cycle from surface to cytoplasm and back, the surface receptor number represents only a fraction of the total receptor number (Lamb *et al.* 1983). To determine whether the lead-treated cells had a decreased number of total transferrin receptors (i.e. cytoplasmic plus surface) or if the observed surface hypoexpression was due to a redistribution of receptors from surface to cytoplasm, we measured the total amount of transferrin receptors in cell lysates from lead-treated and untreated K562 cells. The total amount of transferrin receptors present in the cells was determined by immunoblotting total cell proteins from equal numbers of cells. Gel-resolved proteins were transferred to a Durapore filter, which was incubated with anti-human transferrin receptor antibody. Upon visualization, both lead-treated and untreated cells exhibited a single band of M_r 92 000 present in equal amounts regardless of the treatment, indicating that the total amount of transferrin receptors per cell remained constant throughout lead treatment (Figure 5). These results (i.e. that lead-treated K562 cells exhibited decreased numbers of cell surface receptors, but a constant amount of immunoreactive transferrin receptors) suggested that lead treatment was accompanied by the redistribution of receptor molecules, rather than actual loss of receptors from the cell. We examined the kinetics of receptor cycling in an attempt to discern a mechanism by which the receptors could undergo this redistribution.

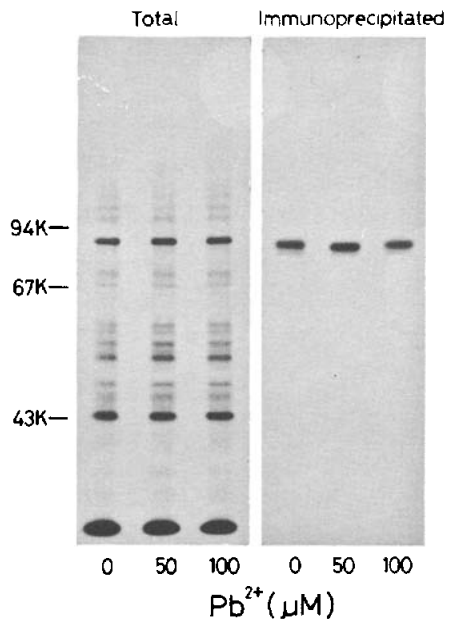

Figure 4. Effect of lead on the biosynthesis of transferrin receptors. K562 cells were cultured with 50 μM, 100 μM or without Pb^{2+} for 48 h and then labeled with [^{35}S]methionine. Left panel: total cell proteins were analyzed by SDS–PAGE. Right panel: transferrin receptors were immunoprecipitated and analyzed by SDS–PAGE.

Kinetics of transferrin recycling

K562 cells were cooled to 4 °C and surface transferrin receptors were labeled using [^{125}I]transferrin.

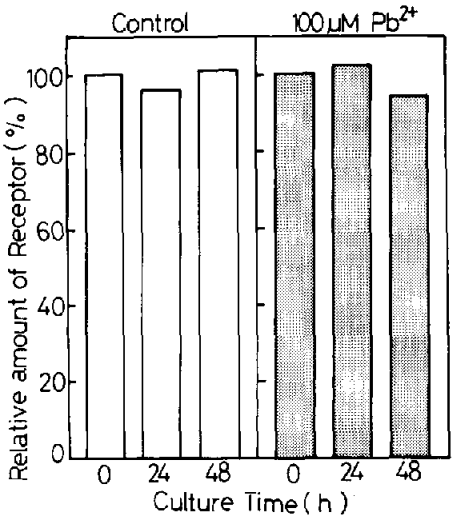

Figure 5. Effect of lead on the total amount of cellular transferrin receptor. The total amount of transferrin receptors was examined by immunoblotting. The intensity of the stained band was quantitated with a densitometer.

Unbound transferrin was washed away and the cells were rapidly warmed to 37 °C by the addition of prewarmed medium. At various times after warming, the radioiodinated transferrin associated with intracellular compartments was determined (Figure 6) using an acid wash technique which specifically removes only surface-located transferrin. The internalized (acid wash-resistant) radioactivity maximized at 5 min (Figure 6). The amount of transferrin detected intracellularly then began to fall—presumably as transferrin receptors recycled back to the cell surface and released transferrin into the medium. When a similar experiment was carried out using cells that had been cultured for 48 h with 100 μM Pb^{2+}, the internalized radioactivity reached a peak after the same period of incubation as untreated cells. However, the amount of internalized transferrin at the peak was reduced to 40% of that in untreated cells, which is consistent with the decrease in surface receptor expression.

In order to compare the rate at which intracellular transferrin is cycling in control cells with that for cells that had been cultured for 48 h with 100 μM Pb^{2+}, cells were incubated at 37 °C for 20 min in serum-free RPMI 1640 medium containing [^{125}I]-transferrin. During this time both surface and internal receptors became occupied. The labeled cells were then washed at 4 °C to free them of unbound ligand and were resuspended at 37 °C by the addition of prewarmed serum-containing medium. At various times after warming, the [^{125}I]-transferrin that remained associated with the cells was measured. The control and lead-treated cells exhibited similar rates of dissociation (Figure 7).

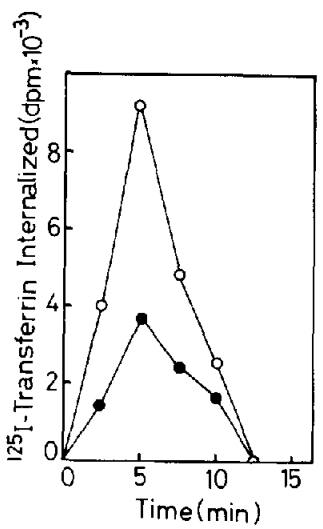

Figure 6. Effect of lead on the rate of transferrin endocytosis. K562 cells were cultured with (●) or without (○) 100 μM Pb^{2+} for 48 h. Surface transferrin receptors were then saturated with [^{125}I]transferrin at 4 °C. After removal of unbound ligand, the cells were shifted to 37 °C and incubated for the indicated times. Surface-bound [^{125}I]transferrin was removed by an acid wash and the amount of internalized transferrin was determined.

Effect of lead on the distribution of functional receptors

When K562 cells were incubated at 37 °C with [^{125}I]transferrin, the amount of cell-associated ligand reached a plateau after 20 min. This plateau represented the steady state between diferric transferrin uptake and apotransferrin release from the cell. Under steady-state conditions, the number of cell-associated ligand molecules should equal the total number of transferrin receptors participating in the cycling pool. Treatment of cells with Pb^{2+} resulted in a decrease in the apparent size of this cycling pool (Table 1), while the rate at which the cycling pool is filled with ligand did not appear to be markedly affected by treatment with Pb^{2+} (data not shown). The ratio of surface receptors to steady-state binding sites also decreased, albeit to a lesser extent (Table 1). These results indicate that the lower number of surface transferrin receptors resulted mainly from a decrease in the cycling pool of functional receptors.

Discussion

Treatment of human erythroleukemia (K562) cells with Pb^{2+} has been shown to decrease iron uptake from transferrin (Figure 1). The reduction of iron uptake was correlated with a decreased concentration of surface transferrin receptors with no change in receptor binding affinity (Figure 3). The close correlation found between the rates of iron uptake and receptor number on the cell surface in cells treated with two concentrations of Pb^{2+} suggests that any other mechanisms of action on iron uptake must be quantitatively unimportant. Such mechanisms include neutralization of intravesicular pH, inhibition of metabolism and interference with transferrin–receptor interaction. The immunoprecipitation of surface transferrin receptors also supports this conclusion (Figure 2).

The expression of transferrin receptors is regulated by complex mechanisms. Previous studies have indicated that iron salts added to the culture medium induce a decline in transferrin receptor synthesis (Louache *et al.* 1984, 1985). Leukemic cell lines (HL-60 and MEL) showed reduced synthesis of the

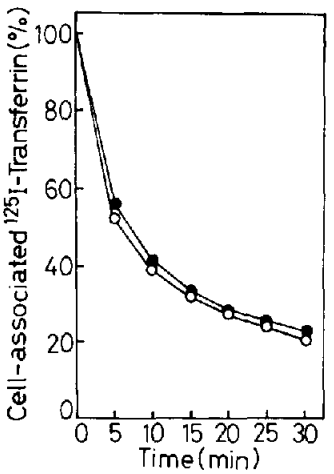

Figure 7. Effect of lead on the rate of release of [^{125}I]transferrin from the cells. K562 cells were cultured with (●) or without (○) 100 μM Pb^{2+} for 48 h. Cells were then incubated with [^{125}I]transferrin at 37 °C for 20 min. Cells were washed at 4 °C to remove unbound transferrin, and then shifted to 37 °C and incubated for the indicated times. Cells were washed at 4 °C and cell-associated radioactivity was determined.

receptor during dimethylsulfoxide-induced differentiation (Enns *et al.* 1988, Mulford & Lodish 1988). Furthermore, we reported diminished biosynthesis of transferrin receptor in K562 cells treated with phorbol ester (Kohno *et al.* 1986). In all of these cases, down-regulation of transferrin receptors was observed. In the present study, however, the down-regulation of the surface receptors in lead-treated K562 cells was not due to the same mechanism since no significant change was detected in the biosynthesis of the receptor (Figure 4).

Studies from several laboratories have documented the existence of a substantial pool of intracellular transferrin receptors (Bleil & Bretscher 1982, Lamb *et al.* 1983). A decrease in the number of surface transferrin receptors in K562 cells after lead treatment might thus be the consequence of either a reduction in the total number of receptors or an accumulation of these receptors in an intracellular compartment. The results of quantitative immunoblotting of transferrin receptors from total cell lysates demonstrated that the relative amount of transferrin receptor was not detectably altered by exposure to Pb^{2+}, indicating that no significant change occurred in the total transferrin receptor pool (Figure 5).

Hunt *et al.* (1984) reported that when K562 cells were induced to differentiate in culture, transferrin receptors detected at the cell surface were reduced in number. They suggested that the reduction of surface receptors was not the result of a decrease in the total number of receptors in the cell but appeared to result from increased internalization. Moreover, Mulford & Lodish (1988) reported that, during terminal differentiation of MEL cells, the number of surface transferrin binding sites per cell decreased dramatically, while immunoblotting studies demonstrated that the total number of transferrin receptors per cell remained constant. They suggested that the increase in the fraction of the receptors localized in the cell interior was due to an altered rate of endocytosis.

May *et al.* (1984) reported that the activation of protein kinase C with phorbol esters resulted in rapid internalization of cell-surface transferrin receptors in human leukemia (HL-60) cells. In the context of this result, it is of interest that Markovac & Goldstein (1988) showed the stimulatory effect of lead on protein kinase C activity. On the other hand, the cytosolic free calcium concentration is one of the possible candidates for the modulator of receptor movement. The interaction between lead and calcium has been investigated in various biological systems (Simons 1986). Exposure to lead enhances calcium uptake in several tissues (Pounds 1984). It, therefore, seems possible that lead causes the internalization of surface transferrin receptors through the stimulation of protein kinase C and/or the increase in calcium uptake.

Table 1. Effect of lead on steady-state transferrin binding

Pb^{2+} (μM)	Steady-state binding (A) (c.p.m./10^6 cells)	Surface binding (B) (c.p.m./10^6 cells)	B/A (%)
0	24350 ± 730	7493 ± 545	30.4
50	18957 ± 765	4611 ± 387	24.3
100	13709 ± 1035	3154 ± 867	23.0

K562 cells were incubated with 50 μM, 100 μM or without Pb^{2+} for 48 h. [^{125}I]transferrin binding at 37 °C for 30 min (steady-state binding) or at 4 °C for 90 min (surface binding) was then determined as described under Materials and methods. Data presented are the mean values from three separate experiments.

We found that steady-state binding of transferrin, which represents the cycling pool of the receptor, was decreased in lead-treated K562 cells (Table 1). Similar phenomena were observed in K562 cells treated with a inhibitor of calcium-dependent protein kinase, trifluoperazine (Hunt & Marshall-Carlson 1986). Furthermore, Sawyer & Krantz (1986) reported that the number of steady-state transferrin binding sites was reduced in Friend virus-infected erythroid cells during maturation. The mechanism by which the number of steady-state binding sites for transferrin were reduced is not known. One explanation for these results is the sequestration of receptors within the cells from the normal flow of receptors from the cell surface through the cell and back to the surface. In this connection, Stein & Sussman (1986) reported that monensin inhibited the recycling of transferrin receptors by trapping them intracellularly. Additional studies would be required in order to determine whether lead causes the sequestration of the receptors by same mechanism as monensin.

Acknowledgments

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