Exposure of K562 Cells to Anti-receptor Monoclonal Antibody OKT9 Results in Rapid Redistribution and Enhanced Degradation of the Transferrin Receptor

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Abstract. When the human erythroleukemia cell line K562 is treated with OKT9, a monoclonal antibody against the transferrin receptor, effects on receptor dynamics and degradation ensue. The apparent half-life of the receptor is decreased by >50% as a result of OKT9 treatment. The transferrin receptor is also rapidly redistributed in response to OKT9 such that a lower percentage of the cellular receptors are displayed on the cell surface. OKT9 treatment also leads to a decrease in the total number of receptors participating in the transferrin cycle for cellular iron uptake. The reduction in iron uptake that results from the loss of receptors from the cycle leads to enhanced biosynthesis of the receptor. Receptors with bound OKT9 continue to participate in multiple cycles of iron uptake. However, OKT9 treatment appears to result in a relatively small increase per cycle in the departure of receptors from participation in iron uptake to a pathway leading to receptor degradation. Radiolabeled OKT9 is itself degraded by K562 cells and this degradation is inhibitable by leupeptin or chloroquine. In the presence of leupeptin, OKT9 treatment results in the enhanced intracellular accumulation of transferrin. Because the time involved in the transferrin cycle is shorter (12.5 min) than the normal half-life of the receptor (8 h), a small change in recycling efficiency caused by OKT9 treatment could account for the marked decrease in receptor half-life. In this paper the implications of these findings are discussed as they relate to systems in which receptor number is regulated by ligand.

PROLIFERATING cells require exogenous iron and acquire it via receptor-mediated endocytosis of the iron-carrier protein transferrin (Tf)[1] (for review see reference 6). Iron uptake via the Tf cycle involves binding of diferric Tf to a specific, high-affinity surface receptor followed by internalization of the receptor ligand complex. Within the cell, the complex encounters an acidic (~pH 5) environment that is instrumental in iron unloading (22, 31). Apo-Tf is then released intact from the cell, and the transferrin receptors (TfR) are reused (2, 9). The Tf cycle shares certain aspects with other systems of receptor-mediated endocytosis but also exhibits distinctive features, including the return of undegraded ligand and receptor to the cell exterior.

In the present study, we describe perturbations of the pathway traversed by the TfR that result from exposure of K562 cells to a monoclonal anti-receptor antibody (OKT9). The monoclonal antibody OKT9 recognizes the human TfR at a site distinct from the ligand binding site and has been used to examine the structural features of the receptor as well as in the chromosomal localization and cloning of the TfR gene (3, 15, 25). We have found that treatment of K562 cells with OKT9 affects receptor distribution and the rates of receptor degradation and biosynthesis. Many of the effects of OKT9 on TfR dynamics in K562 cells resemble phenomena observed in other endocytic systems including what is thought to be physiologic modulation of receptor function. Accordingly, we believe that certain implications of our experiments may be generally applicable to receptor dynamics.

[1] *Abbreviations used in this paper:* Tf, transferrin; TfR, Tf receptor.

Materials and Methods

Cells and Chemicals

K562, a human erythroleukemia cell line, was grown in RPMI 1640 with 25 mM Hepes containing 10% fetal bovine serum, penicillin, and streptomycin (growth medium). Cells were maintained at densities of 2–6 × 10^5/ml at 37°C in 5% CO_2 incubator.

Human Tf (Calbiochem-Behring Corp., San Diego, CA) was made diferric by published procedures (11). Anti-TfR monoclonal antibody OKT9 was prepared from ascities fluid using DEAE Affigel blue (Bio-Rad Laboratories, Richmond, CA) as previously described (1). It was further purified by high-pressure liquid chromatography by passage through a TSK G3000 SW column (LKB Instruments, Gaithersburg, MD) with elution in phosphate-buffered saline (PBS) at 0.5 ml/min. 0.25-ml fractions were collected and the IgG peak was detected by absorbance at 280 nm. Monoclonal antibody B3/25 was obtained from Boehringer-Mannheim Diagnostics (Houston, TX) and exhaustively dialyzed against PBS.

Tf and IgG were iodinated by Enzymobeads (Bio-Rad Laboratories) according to the supplier's instructions. After from 30 min to 1 h of iodination at room temperature the reaction mixture was passed through a Sephadex G-25

column (Pharmacia PD 10) that had been equilibrated with 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4 (for Tf), or PBS (for OKT9), and the protein peak was recovered. Specific activities ranged from 240 to 2,400 cpm/ng protein, depending on the nature and amount of protein iodinated. Tf radiolabeled with ^{59}Fe was prepared by methods previously published (11).

Hemin (ferric protoporphyrin IX) was purchased from Porphyrin Products, Inc. (Logan, UT). Just before use, hemin was dissolved in 0.2 M Na_2CO_3/$NaHCO_2$, pH 10.7, and the pH was adjusted to 7.4 with 1 M HCl. This solution was filtered through a 0.45-μm filter before addition to cells.

Degradation

Rates of degradation of the TfR were assessed using metabolically labeled receptor. Metabolic labeling was achieved by incubating K562 cells (5×10^5/ml) in methionine-free RPMI 1640 medium containing 10% dialyzed fetal bovine serum and 50 μCi/ml [^{35}S]methionine (1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL). After 2 h in a 37°C incubator, cells were washed three times with K562 growth medium and returned to the incubator. Receptor degradation experiments were begun 45 min later, when the cell suspension was divided and the desired additions were made. Immediately and at intervals thereafter, samples were removed, and the content of radiolabeled TfR was determined by cell lysis, immunoprecipitation, gel electrophoresis, and autoradiography. At each time point, cells were pelleted and lysed in 1 ml ice-cold 1% Triton X-100, 0.5% sodium desoxycholate, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.1% bovine serum albumin ([BSA] lysis buffer). Immediately, the solubilized cells were made 0.1 mM in phenylmethylsulfonyl fluoride, 0.5 trypsin inhibitor units (TIU)/ml in aprotinin (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml in leupeptin (Boehringer Mannheim Diagnostics). After 30 min on ice, the samples were centrifuged at 13,000 *g* for 10 min. The supernatant fluid was removed and stored at −70°C until all time point samples were collected.

Immunoprecipitation was accomplished using an IgG fraction prepared from the plasma of a goat injected with TfR purified from K562 cells by affinity chromatography on Tf-Sepharose. In preliminary experiments it was established that the presence of OKT9 did not affect immunoprecipitation of the human TfR by the goat IgG (data not shown). In some experiments, 25 μg goat anti-receptor IgG was added to 0.5 ml cell lysate supernatant fluid whereas in others 50 μl of Sepharose CL6B (Pharmacia Fine Chemicals) containing 50 μg covalently bound goat anti-receptor IgG were added. In the former case, precipitation was achieved using protein A-Sepharose (Pharmacia Fine Chemicals) coated with rabbit anti-goat IgG (Cappel Laboratories, Cochranville, PA). In both cases, the resins were tumbled for 90 min at 4°C before being centrifuged and washed three times with 1 ml lysis buffer lacking serum albumin. The resin pellets were then heated for 5 min at 95°C in electrophoresis sample buffer. The released proteins were electrophoresed on 12.5% polyacrylamide gels cross-linked with Acrylaide and bonded to Gel Bond (FMC Corp., Chicago, IL) using the buffer system of Laemmli (16). After fixation, gels were impregnated with 1 M sodium salicylate and dried in a 60°C oven. Dried gels were exposed to Kodak XAR 5 film at 70°C. The amount of radiolabeled TfR present in each sample was quantitated from the resultant autoradiograph using a Hoefer scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA) and peak integration.

Biosynthesis

The rate of biosynthesis of the TfR was also assessed by immunoprecipitation, gel electrophoresis, and autoradiography. In these biosynthetic experiments, K562 cells (1×10^6/ml) were incubated for 30 min in RPMI 1640 medium containing one-tenth the normal amount of methionine, 10% fetal bovine serum, and 0.2 mCi/ml [^{35}S]methionine. Analyses of the radiolabeled cells were accomplished essentially as described above except that OKT9 IgG (5 μg) and Protein A-Sepharose (Pharmacia Fine Chemicals) were used for immunoprecipitation. The treatments shown to affect TfR biosynthesis did not alter [^{35}S]-methionine incorporation into total cellular protein as judged by 10% trichloroacetic acid precipitation of radioactivity.

Soluble Receptor Assay

K562 cells (1.2×10^6) were solubilized in 0.5 ml ice-cold 0.5% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 TIU/ml Aprotinin, 2.5 μg/ml ^{125}I-Tf. After 45 min on ice, 0.5 ml 60% saturated ammonium sulfate (adjusted to pH 7.6 with Tris base) was added, and this mixture was incubated for 15 min at room temperature. The ammonium sulfate-precipitated receptor-ligand complex was recovered by filtration on a Whatman GF/C filter (Whatman Laboratory Products Inc., Clifton, NJ) which was subsequently washed twice with 3 ml 30% saturated ammonium sulfate. Nonspecific binding was assessed by inclusion of 250 μg of unlabeled human diferric Tf in the solubilization mixture. The presence of OKT9 did not affect the values obtained using this soluble receptor assay (data not shown).

Binding and Uptake Studies

Binding studies with either ^{125}I-Tf or ^{125}I-OKT9 were carried out in RPMI 1640 with 25 mM Hepes, pH 7.4, containing 0.1% BSA (assay medium). Binding experiments were performed at ^{125}I-Tf concentrations of ≥10 μg/ml. Under these conditions no effect of the presence of OKT9 on the amount of ^{125}I-Tf bound was seen. Nonspecific binding was determined by the addition of a 100-fold excess of the unlabeled material of interest. Cell-associated radioactivity was separated from unbound material by layering of 200 μl cell suspension over 150 μl of either 1 M sucrose in PBS, pH 7.2–7.4, or 150 μl dibutyl phthalate (Aldrich Chemical Co., Milwaukee, WI) in 400-μl microfuge tubes (Bio-Rad Laboratories). The cells were pelleted by centrifugation in a Beckman microfuge (Beckman Instruments Inc., Palo Alto, CA) for 1 min. The tips containing cell pellets were cut and their content of ^{125}I was determined in a Packard Auto Gamma 5060 gamma-counter (Packard Instrument Co., Inc., Downers Grove, IL). In untreated cells, the nonspecific binding/uptake always represented <15% of total cell-associated radioactivity and was usually ~5%. The absolute levels of nonspecific binding/uptake were not significantly changed by any of the treatments employed.

Results

Effect of Anti-receptor Antibody on the Degradation of the TfR

Treatment of K562 cells with mouse monoclonal IgG (OKT9) against the TfR resulted in a decrease in the apparent half-life of the TfR from ~8 to ~4 h. This effect of OKT9 on receptor degradation was assessed by pulse-chase experiments using cells labeled metabolically with [^{35}S]methionine (Fig. 1) or in chase experiments after surface radioiodination (data not shown). The apparent half-life of total cellular proteins in K562 cells as judged by the loss of 10% trichloroacetic acid–precipitable radioactivity was found to be ~19 h. The presence of saturating levels of human Tf did not affect the degradation rate of the TfR (18), nor did added human Tf alter the enhanced degradation rate seen in OKT9-treated cells. Another monoclonal antibody against the human TfR (B3/25)

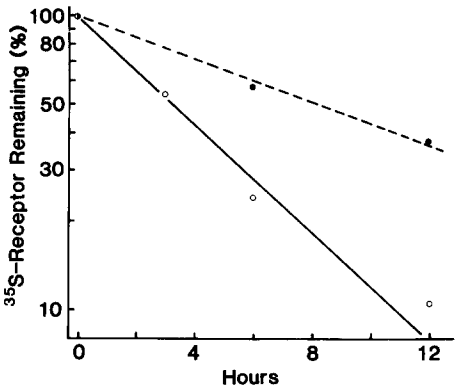

Figure 1. Effect of OKT9 treatment on the degradation of the TfR. K562 cells were metabolically labeled with [^{35}S]methionine for 2 h at 37°C as described in Materials and Methods. After being washed free of unincorporated [^{35}S]methionine and incubated for an additional 45-min at 37°C, the cell suspension was divided, and OKT9 (5 μg/ml) was added to half of the cells. At the indicated times thereafter, samples were removed and their content of radiolabeled receptor was determined by immunoprecipitation, gel electrophoresis, autoradiography, and densitometry as described in Materials and Methods. Values are expressed as percentages of the value obtained at the time of antibody addition. Shown are the first-order decay curves for untreated (●) and OKT9 treated (○) cells.

had effects analogous to those of OKT9 on receptor degradation whereas an unrelated monoclonal antibody (OKT3) did not. To determine whether receptor shedding might account for the OKT9 effect, we solubilized and immunoprecipitated K562 cells together with their incubation medium rather than the washed cells alone. Here, a similar OKT9-mediated decrease in receptor half-life was observed, arguing against a shedding of TfR in response to OKT9 treatment as an explanation of the OKT9 effect. When the OKT9 IgG was itself radioiodinated and incubated with K562 cells at 37°C, the ^{125}I-IgG became associated with the cells at at a level approximately twofold higher than the binding capacity of the cell surface (Fig. 2). Between 2 and 3 h of incubation the level of cell-associated ^{125}I-IgG began to decline. All of the loss of cell-associated radioactivity could be accounted for in 10% trichloroacetic acid–soluble radioactivity, suggesting that proteolysis occurred. The loss of radioactivity from the cells and appearance of acid soluble radioactivity were prevented by inclusion of the protease inhibitor leupeptin. In other experiments, we found that chloroquine also inhibited ^{125}I-OKT9 degradation (data not shown).

Effect of Anti-receptor Antibody on the Cellular Content and Biosynthesis of the TfR

The antibody-mediated increase in TfR degradation would be expected to result in a corresponding decrease in the steady state content of cellular TfR. However, when TfR binding sites were measured in detergent-solubilized cells after lengthy treatment with OKT9, the level of TfR was found to be decreased by <30% (Fig. 3) despite a ≥50% decrease in receptor survival time. The relatively smaller drop in total receptor number led us to assess directly the biosynthetic rate of the receptor in OKT9-treated cells. The results shown in Fig. 4 demonstrate that treatment with OKT9 resulted in a greater than twofold increase in the rate of TfR biosynthesis. In contrast, cells treated with saturating levels of human Tf exhibited a rate of biosynthesis that is decreased to <50% of the control value. This human Tf effect has been previously documented by our laboratory and appears to be due to decreased levels of TfR mRNA resulting from transcriptional modulation (18, 20, 21). A decrease in receptor biosynthesis was also seen when cells were treated with other iron-supply-

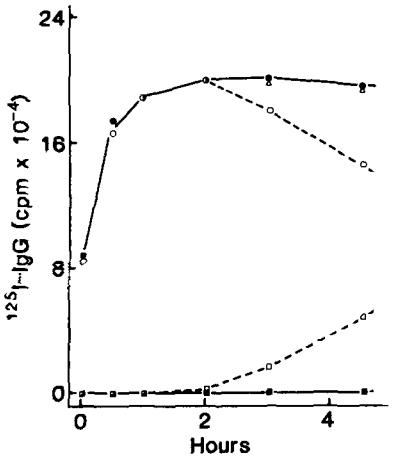

Figure 2. Uptake and degradation of ^{125}I-OKT9. K562 cells were washed into ice-cold assay medium. To the suspension of cells (1 × 10^6 cells/ml), 1 μg/ml ^{125}I-OKT9 was added and the cell suspension was incubated at 4°C for 30 min before rewarming. Leupeptin (0.1 mg/ml) was added to these incubations where indicated (●, ■). Cells were warmed to 37°C in a 5% CO_2 incubator for the indicated times when samples were removed and cell-associated ^{125}I-OKT9 (●, ○) was determined as described in Materials and Methods. To determine the extent of degradation of ^{125}I-OKT9 samples of the cell suspension were added to an equal volume of ice-cold 20% trichloroacetic acid, 4% phosphotungstic acid. After 30 min on ice, this material was centrifuged and the amount of acid-soluble radioactivity was determined (■, □). For the cultures not receiving leupeptin, cell-associated and acid-soluble radioactivity have been summed (△) to demonstrate that all radioactivity lost from the cells can be accounted for as degraded ^{125}I-OKT9. To account for acid-soluble radioactivity that was not due to cellular uptake, flasks containing the appropriate media and ^{125}I-OKT9 were incubated without K562 cells, and corresponding values were subtracted.

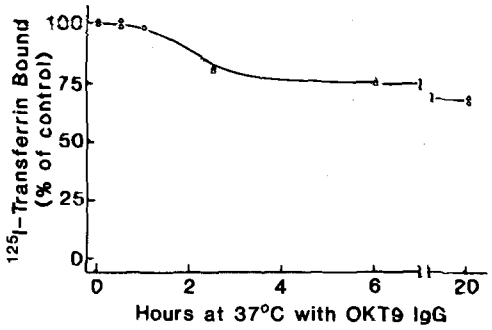

Figure 3. Effect of OKT9 treatment on cellular content of transferrin receptors. OKT9 (2 μg/ml) was added to K562 growth cultures such that the indicated treatment times were attained. The cells (2 × 10^6) were centrifuged, lysed in detergent, and the ^{125}I-Tf binding capacity of the solubilized cells was determined as described in Materials and Methods. Values are expressed as percentages of the value obtained with cells having never been incubated at 37°C with OKT9.

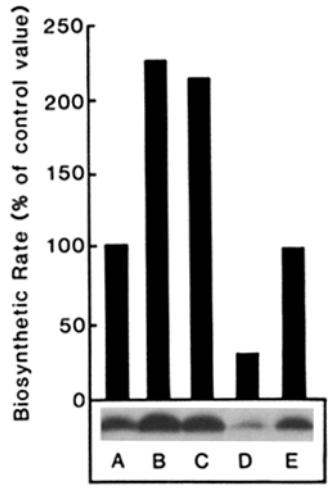

Figure 4. Effect of various treatments on rate of biosynthesis of the TfR. K562 cells were incubated for 6 h at 37°C with no additions (lane *A*), 5 μg/ml OKT9 (lane *B*), 5 μg/ml B3/25 (lane *C*), 40 μg/ml human diferric Tf (lane *D*), or 5 μg/ml OKT9 plus 40 μg/ml human diferric Tf (lane *E*). Cells (1 × 10^6) from each incubation were centrifuged, and the rate of receptor biosynthesis was determined by pulse-labeling with ^{35}S-methionine, immunoprecipitation, electrophoresis, and autoradiography as described in Materials and Methods. The upper portion of the figure displays the results of quantitative densitometry of the autoradiograph. These data are expressed as percentages of the value obtained for control cells (lane *A*).

ing substances such as ferric ammonium citrate (21) or hemin (24). Simultaneous treatment with human diferric Tf and OKT9 (Fig. 4) has the effect of preventing the enhanced biosynthesis of TfR seen in cells treated with OKT9 alone. To evaluate whether the biosynthetic effect of OKT9 might be related to iron deprivation, hemin was employed as an iron source. We have recently shown that hemin can serve as a very efficient source of chelatable iron that can depress TfR biosynthesis. In the presence of hemin, OKT9-mediated reduction of total cellular TfR is considerably more pronounced (Table I), owing to lower rates of receptor biosynthesis under these conditions. These results suggest that the OKT9 effect on TfR biosynthesis is probably a secondary effect resulting from a decrease in iron within the regulatory iron pool.

Effect of Anti-receptor Antibody on the Tf Cycle

The effect of antibody on a single Tf cycle was examined. After ^{125}I-Tf was prebound to the cell surface at 4°C and unbound ^{125}I-Tf was removed, cells were warmed to 37°C (Fig. 5). There was no detectable effect of OKT9 treatment

Table I. Effect of OKT9 and/or Hemin on the Number of Cellular TfR

Treatment	^{125}I-Tf Bound	% of control
	cpm	
None	37,400	100
OKT9	26,462	70.7
Hemin	35,676	95.7
OKT9 + Hemin	18,588	49.7

K562 cells were treated as indicated with OKT9 (2 μg/ml), hemin (50 μM), or OKT9 plus hemin. After 6 h at 37°C in a 5% CO_2 incubator, 1.4 × 10^6 cells were subjected to the soluble TfR assay described in Materials and Methods. Values shown represent the average of duplicate determinations.

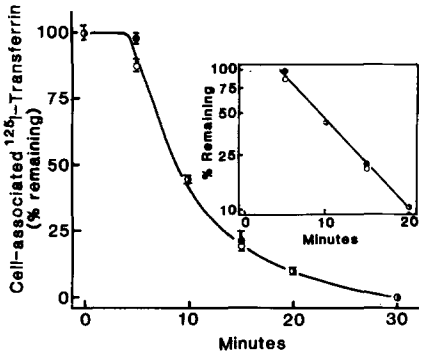

Figure 5. Effect of OKT9 treatment on efflux of cellular transferrin. K562 cells were cooled to 4°C, centrifuged, and resuspended at 3.5 × 10^6/ml in assay medium. After the addition of 1.25 μg/ml OKT9 (●) or no addition (○), the cell suspensions were warmed to 37°C for 45 min. After cooling to 4°C, 5 μg/ml of ^{125}I-Tf was added, and the cell suspension was incubated at 4°C for 15 min. The cells were washed twice at 4°C to remove unbound ligand and resuspended in assay medium containing 5 μg/ml unlabeled human diferric Tf to prevent rebinding of radiolabel. OKT9 (1.25 μg/ml) was restored to those cell suspensions that had been pretreated with the antibody. The suspensions were warmed to 37°C, and, at the indicated times, samples (0.2 ml) were removed and cell-associated radioactivity was assessed as described in Materials and Methods. Values are expressed as percentages of the initial value, and standard errors are shown. The inset provides a semilogarithmic plot of data showing that single exponential functions define the loss of cell-associated ^{125}I-Tf in both control (○) and OKT9 treated (●) cells.

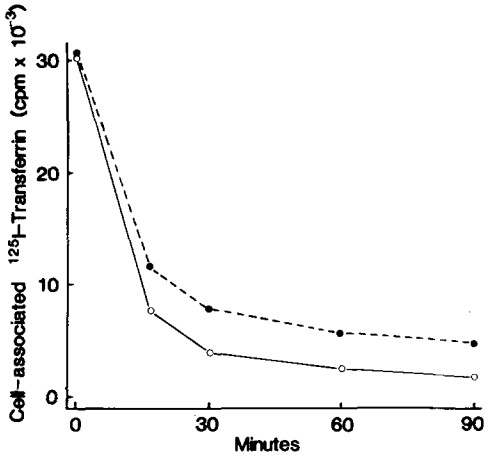

Figure 6. The influence of OKT9 and leupeptin on the retention of cellular ^{125}I-Tf. K562 cells were cooled to 4°C, centrifuged, and resuspended at 4°C at 5 × 10^6/ml in in assay medium in the presence of leupeptin (0.1 mg/ml). Suspensions then were made 20 μg/ml in ^{125}I-Tf and warmed to 37°C. After 15 min of warming, either OKT9 (1 μg/ml) (●) or an equivalent volume of PBS (○) was added. The cell suspensions were kept at 37°C for an additional 75 min. Cells were then cooled to 4°C and washed twice in ice-cold assay medium before being resuspended at 5 × 10^6/ml in assay medium containing 80 μg/ml unlabeled human diferric Tf and 0.1 mg/ml leupeptin. OKT9 was included in the washes and resuspension medium as indicated above. The cell suspensions were then once again warmed to 37°C for the indicated times when samples (0.2 ml) were removed and cell-associated radioactivity was assessed as described in Materials and Methods. All measurements were performed in quadruplicate, and the values obtained varied from the mean no more than ±5%.

on the lag time (~4 min), rate ($t_{1/2}$ = 5 min), or extent (~98%) of the exponential loss of cell-associated ligand.

Because the TfR participates in many cycles of iron uptake in its lifetime, a relatively small increase in the percentage of the TfR being degraded per cycle could be responsible for the enhanced receptor degradation seen in OKT9 treated cells. Since Tf does not dissociate from TfR in the Tf cycle (2, 6, 9) OKT9 might also be expected lead to intracellular accumulation of ^{125}I-Tf. If a small percentage of the receptor ligand complexes engaged in each Tf cycle were to leave the cycle and remain within the cell pending degradation, this would be detected only if ligand molecules that had departed the cycle were allowed to accumulate over multiple cycles. An experiment of this nature is shown in Fig. 6. In OKT9-treated cells ~15% of the radiolabeled ligand that had accumulated in a 90-min incubation with ^{125}I-Tf was retained within the cell during the second 90-min incubation with excess unlabeled ligand in the medium. In control cells <7% of the ^{125}I-Tf was found in this nonexchangeable pool. This experiment was performed in the presence of the protease inhibitor leupeptin to prevent proteolytic breakdown of the ^{125}I-Tf. The levels on nonexchangeable ligand in both control and OKT9-treated cells were significantly decreased by omission of leupeptin (data not shown). These results together with those in Fig. 2 suggest that OKT9 enhances movement of the antibody receptor-ligand complex from the normal Tf cycle to an intracellular pathway that terminates in leupeptin-sensitive degradation of receptor-associated OKT9 and of receptor-associated ligand.

When K562 cells are incubated at 37°C with ^{125}I-Tf, the

amount of cell-associated ligand soon reaches a plateau that represents the steady state between endocytosis of diferric Tf and exocytosis of apo Tf (2, 9). In the course of the normal Tf cycle, this provides an estimate of the number of TfR involved in iron delivery, i.e., this level of cell-associated ^{125}I-Tf serves as a measure of the cycling pool of TfR. Treatment of cells with OKT9 results in a decrease in the apparent size of this cycling pool (Fig. 7). Consistent with the results in Fig. 5, the rate at which the cycling pool is filled with ligand does not appear to be markedly affected by treatment with OKT9.

The results presented above demonstrate that most cell-associated Tf is released when OKT9 is present (Figs. 5 and 6) but that, after treatment with OKT9, cells increase their accumulation of Tf during multiple cycles (Fig. 6). To confirm that continued uptake of iron via the Tf cycle is occurring in the presence of Tf and OKT9, uptake of ^{59}Fe from [^{59}Fe]Tf was compared in control and OKT9-treated cells (Fig. 8). The uptake rate (slope) in OKT9-treated cells was decreased ~20% as compared with that seen in the control cells. These results demonstrate that the Tf cycle is operative in OKT9-treated cells and that iron uptake is decreased commensurate with the reduction shown in Fig. 7 of the number of TfR within the cycling pool. These results do not rule out the possibility that continued iron uptake in the presence of OKT9 is due to entry of new receptors into the system. The inset of Fig. 8 argues against this possibility. Here cells were incubated in the presence of ^{125}I-OKT9 under conditions used in ^{59}Fe uptake experiments. As receptor-bound OKT9 does not readily dissociate from K562 cell membranes at 37°C (data not shown), this inset can be interpreted to indicate that no significant number of receptors without bound OKT9 appears at the cell surface in the period (2–3 h of OKT9 treatment) during which ^{59}Fe uptake was measured. Thus, it appears that most of the receptors with bound OKT9 continue to participate in the Tf cycle and continue to mediate iron uptake.

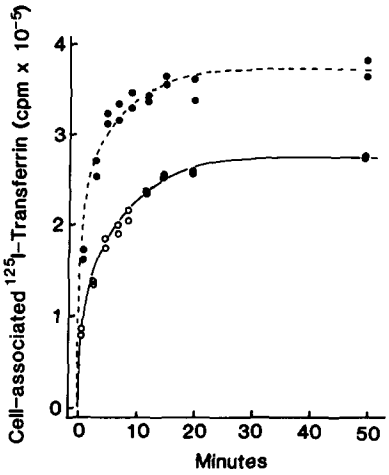

Figure 7. Effect of OKT9 treatment on the cycling pool of TfR. K562 cells were treated with 2 μg/ml OKT9 for 1 h at 37°C in a 5% CO_2 incubator. The cells were then centrifuged and resuspended in assay medium at 5.5 × 10^6/ml. The cell suspension were warmed to 37°C and added to an equal volume of prewarmed assay medium containing 20 μg/ml ^{125}I-Tf. Duplicate samples (0.2 ml) were taken at the indicated times and the cell-associated radioactivity was assessed as described in Materials and Methods. Cell-associated radioactivity for OKT9-treated (○) and for untreated (●) cells is shown. The plateau value representing the steady state between diferric Tf uptake and apo-Tf release has been taken as a measure of the number of TfR participating in the cycling pool.

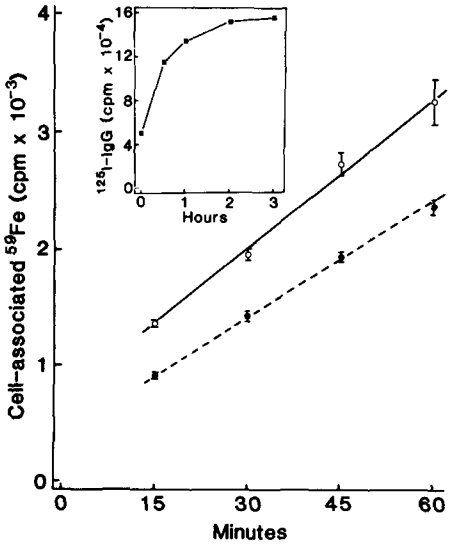

Figure 8. Effect of OKT9 treatment on iron uptake in K562 cells. K562 cells were treated where indicated (●) with 1 μg/ml OKT9 for 2 h at 37°C in a 5% CO_2 incubator. Cells were then cooled to 4°C, centrifuged, and resuspended at 2.8 × 10^6/ml in ice-cold assay medium. OKT9 (1 μg/ml) was re-added to the appropriate cell suspensions. ^{59}Fe-Tf 9,000 cpm/μg) was then added at a concentration of 40 μg/ml, and after 20 min at 4°C the cells were warmed to 37°C. At the indicated times cell-associated ^{59}Fe was assessed as described in Materials and Methods. A comparison was made to cells not treated with OKT9 (○). Standard errors are shown. In the inset is shown cell-associated ^{125}I-OKT9 in a parallel experiment in which K562 cells were incubated at 37°C with 1 μg/ml ^{125}I-OKT9. At the indicated times cells were cooled to 4°C, centrifuged, and resuspended in assay medium at 4 × 10^6/ml. Cell-associated radioactivity was assessed as described in Materials and Methods. The zero-time value represents the value for cell-associated ^{125}I-OKT9 obtained with unwarmed cells.

Effect of Anti-receptor Antibody on the Distribution of the TfR

It is clear from the above data that both cycling pool receptors and total cellular receptors are reduced by OKT9 treatment. Accordingly, we addressed the question of whether this reduction was proportionally reflected in both cell surface and intracellular receptors (Fig. 9). Receptors disappeared from the cell surface (Fig. 9*A*) at a rate exceeding reduction in the cycling pool (Fig. 9*B*). In the inset of Fig. 9*B*, it can be seen that the reduction in the number of cell surface receptors can be described by two exponential functions, the slower of which resembles the rate of reduction in the size of the cycling pool. The effect of the initial rapid component is a redistribution of the cycling pool with the result being less representation on the cell surface. In a number of experiments, we observed that OKT9 induced a 40–60% decrease in the fraction of the cycling pool receptors that are displayed on the cell surface.

Discussion

Receptor-mediated endocytosis provides the means for internalization of ligands and receptors in many systems (19, 26). The events that follow internalization and the ultimate fate of the ligand and receptor molecules are qualitatively different in various endocytic systems. In certain cases, ligands are

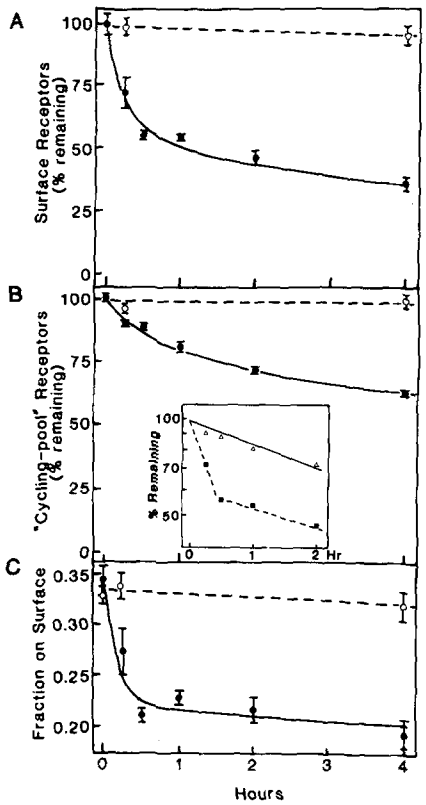

Figure 9. Effect of OKT9 treatment on surface and cycling pool receptor numbers. K562 cells (4×10^5/ml) were treated with 1 μg/ml OKT9 (●) for the indicated times in a 5% CO_2 incubator at 37°C. Control cells (○) received an equivalent volume of PBS. For zero time points, cells were cooled before the addition. The additions were staggered so that all cells were removed from incubation at the same time. The cells were then cooled to 4°C, centrifuged, and resuspended at 4×10^6/ml in assay medium containing ^{125}I-Tf (20 μg/ml). OKT9-treated cells had antibody re-added. Surface receptors (*A*) were determined by maintaining cells at 4°C for 45 min before assessment of cell-associated radioactivity as described in Materials and Methods. Cycling pool receptors (*B*) were determined by warming cells for 45 min to 37°C and then cooling to 4°C to measure total cell associated radioactivity. The *B* inset shows surface (■) and cycling pool (△) receptor numbers for OKT9-treated cells plotted semilogarithmically. The ratio of the values obtained for surface and cycling pool receptors is plotted in *C* for control (○) and OKT9 treated (●) cells. Standard errors are shown.

degraded and their receptors reused whereas, in others, both ligand and receptor are destroyed. During Tf-mediated iron uptake, both undegraded Tf molecules and TfR are overwhelmingly returned to the cell exterior (6). There is compelling evidence from many endocytic systems that the degree of recycling of receptor and ligand is well regulated. There exist multiple possible mechanisms by which receptor functions might be modulated. Alteration of the location of receptors within the cell may render them more or less available without changing the total cellular content of receptor molecules. The actual number of receptors can be altered by changes in rates of biosynthesis and/or degradation. Alternatively, function may be directly modulated either by changes in the affinity of the receptor for ligand or through effects on the transduction of signals that arise from ligand binding.

Treatment of K562 cells with OKT9 results in two major effects related to receptor dynamics. Such treatment leads to an ~50% reduction in the apparent half-life of the TfR and the receptors engaged in iron uptake are redistributed. The enhancement of receptor degradation leads to a reduction in the steady state cellular content of TfR by ~30%. However, this reduction in TfR content is not as pronounced as would be expected based upon the magnitude of the effect on receptor half-life. This apparent discrepancy is reconciled by the finding that OKT9 treatment also leads to an approximate doubling of the rate of TfR biosynthesis. This effect of anti-receptor antibody on receptor biosynthesis is probably mediated via an intracellular regulatory pool of iron. Iron supplied by human diferric Tf through the TfR or by hemin via a pathway not involving the TfR was shown to result in suppression of the OKT9 effect on TfR biosynthesis. The mechanism by which OKT9 treatment leads to enhanced receptor degradation remains obscure. It is possible that receptor cross-linking by divalent IgG at some point in the process is involved. Receptor cross-linking has been implicated in endocytosis mediated by the IgG Fc receptor (19). However, note that ligands (e.g., insulin, epidermal growth factor) not thought to be capable of direct receptor cross-linking can nonetheless mediate receptor down regulation.

Treatment with OKT9 results in a reduction in the number of TfR that are participating in iron uptake. This loss of receptors from the cycling pool leads to a corresponding decrease in ^{59}Fe accumulation from [^{59}Fe]Tf. Trowbridge and Domingo (29) have reported that the antibody B3/25 (that like OKT9 does not block Tf binding to the TfR) can inhibit human tumor cell growth in nude mice. One possibility raised by these authors was that B3/25 might interfere in some way with tumor cell iron uptake. Our results with OKT9 agree with this hypothesis.

Although OKT9 leads to a decrease in the number of total and cycling TfR and thus reduces iron uptake, it is equally clear that receptors with bound OKT9 continue to cycle and mediate iron uptake. The rate of iron uptake in antibody treated cells is decreased only ~20%, a reduction consistent with the measured decrease in the cycling pool of TfR. Moreover, the kinetic parameters that define the Tf cycle do not appear to be significantly altered by OKT9 treatment. Radiolabeled OKT9 was employed to show that "new" receptors (i.e., those that lack bound OKT9) were not responsible for the continued uptake of iron. The reduction in the size of the TfR cycling pool in OKT9-treated cells may reflect movement of TfR from the pathway involved in iron uptake to a pathway ultimately leading to receptor degradation. Taken collectively, our data suggest that enhanced degradation of the TfR results from a relatively small decrease in recycling efficiency.

As the TfR participates in many cycles of iron uptake in its lifetime, small decreases in recycling efficiency per cycle would have profound effects on receptor half-life, assuming these decreases in cycling efficiency result in a movement of TfR into a pathway committed to degradation (Table II). We have calculated a cycle time of 12.5 min for Tf using the measured rate of iron uptake and the measured number of TfR in the cycling pool. If 98.2% of these receptors normally returned per cycle and the remaining receptors were committed to degradation, the observed receptor half-life of 8 h would result. A decrease in per cycle efficiency from 98.2 to 96.5% would decrease the half-life by 50% to 4 h. Were the efficiency to drop to 86.6% the half-life would fall to only 1 h. The

Table II. Effect of Altering the Theoretical Recycling Efficiency on Receptor Half-life

Theoretical recycling (efficiency per cycle)	Resultant receptor half-life
%	
100	∞
99.1	16 h
98.2	8 h
96.5	4 h
86.6	1 h
50.0	12.5 min

The above calculations are based on a first-order decay function for the TfR. From the measured rate of iron uptake and the measured number of participating receptors, a cycle time of 12.5 min has been calculated. The above calculation of hypothetical receptor half-life assumes that receptors must leave the cycling pool to be degraded and that those that leave the cycling pool are degraded.

shorter the cycle time relative to the normal half-life of a receptor, the more marked would be the effects of rather subtle changes in per cycle recycling efficiency. This perspective is based on the behavior of the entire population of TfR involved in iron uptake. In OKT9-treated cells, the population of TfR molecules appears to recycle with an efficiency only slightly decreased from that seen in untreated cells. If the population of TfR is heterogeneous with regard to the nature of OKT9 binding, or if the nature of OKT9 TfR interaction can change with time, then recycling of a subpopulation of TfR may be more markedly affected. Nonetheless, this must be manifested in such a way as to result in only a small decrease in the recycling probability within the TfR population in OKT9-treated cells.

Perhaps the most common effect observed upon treatment of cells with various anti-receptor antibodies is a reduction in cell surface receptor number. This has been previously reported for the receptors for insulin (5, 23, 28), epidermal growth factor (4), mannose-6-phosphate (30), and Tf (7). We have observed a similar phenomenon regarding the TfR in K562 cells treated with phorbol myristic acid or upon addition of human diferric Tf (10). We have observed that neither the OKT9-mediated redistribution described here nor the phorbol myristic acid effect is additive to the human Tf-induced reduction in surface receptors. It is possible that the mechanism involved in all of these may be similar. We have also observed that incubations of cells at 37°C without a 5% CO_2 atmosphere appeared to lead to a "spontaneous" decrease in surface receptors, whereas surface receptor number on cells maintained inside an incubator remained relatively constant. The reason for this effect remains obscure but the observation demonstrates the need to carefully scrutinize even seemingly routine experimental manipulations. This phenomenon may account in part for the lack of consensus that exists regarding constitutive versus ligand-induced cycling of the TfR (10, 32).

The OKT9-mediated loss of TfR from the cycling pool was accompanied by an increase in ^{125}I-Tf that remained cell associated during multiple cycles of endocytosis, i.e., ^{125}I-Tf that did not exchange with extracellular unlabeled ligand in the presence of the protease inhibitor leupeptin. This suggests that it is a complex of antibody, receptor, and ligand that is removed from participation in iron uptake. In both control and OKT9-treated cells, omission of the leupeptin in this experiment resulted in less cell-associated radioactivity in this nonexchanging pool. Degradation of cell-associated ^{125}I-OKT9 also exhibited sensitivity to leupeptin. Although not unambiguously shown by these experiments, lysosomal degradation of ^{125}I-OKT9 and ^{125}I-Tf would be consistent with the leupeptin sensitivities of both OKT9 degradation and the OKT9-mediated ^{125}I-Tf accumulation. That chloroquine also inhibits ^{125}I-OKT9 degradation is in accord with this hypothesis.

In the course of submission and revision of this manuscript Lesley and Schulte (17) published a paper that supports some of the results that we present here. These authors report that treatment of mouse lymphoma cells with monoclonal antibodies directed against the murine TfR reduces cell surface expression of the TfR and enhances degradation of surface radioiodinated TfR.

Ligands of the receptors for insulin and epidermal growth factor result in enhancement of receptor degradation (8, 12–14, 27). Insulin also appears to induce redistribution of its receptor (13). Although the molecular mechanism(s) involved in these phenomena remain obscure, the phenomena themselves resemble the effects reported here of OKT9 on the TfR of K562 cells. It is unclear as to whether these observations using a monoclonal antibody have a relationship to ligand mediated down-regulation. This determination will require additional insight into the molecular signalling events that distinguish the pathways traversed by the TfR from those involved in down regulation.

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