Intact Transferrin Receptors in Human Plasma and Their Relation to Erythropoiesis

By Helmut A. Huebers, Yves Beguin, Pensri Pootrakul, David Einspahr, and Clement A. Finch

Intact transferrin receptor molecules complexed with transferrin were found in human plasma. The concentration of receptors was determined by an enzyme-linked immunosorbent assay that uses polyclonal antibodies. The mean concentration of 8,279 μg/L in 56 normal adults appears to be unrelated to age or sex. Additional receptor measurements were performed on plasmas from 260 subjects with erythropoietic disorders. Decreased concentration of plasma receptors was found in patients with erythroid hypoplasia and increased numbers in those with erythroid hyperplasia. Ferrokinetic measurements of erythropoiesis were compared with numbers of receptors in 148 subjects, and a close correlation was found ($r = .86$). Both sets of values, measured in different conditions and expressed in relation to normal, were consistent with expected values. Receptor values were unproportionally increased only in conditions of iron deficiency. It is concluded that plasma receptors have a constant relationship to tissue receptors, and their number in most instances reflects the rate of erythropoiesis.

IRON DELIVERY is accomplished by a plasma protein, transferrin, which delivers iron to the cell membrane where a receptor enables transferrin to enter the cell and release its iron.[1] In the past, ferrokinetic techniques have been used to characterize iron delivery, particularly to the erythron.[2] Recently Kohgo et al[3] demonstrated the presence of circulating material in the human plasma, presumably transferrin receptors or their degradation products, which reacted with monoclonal transferrin receptor antibodies. They further reported that the amount of receptor material appeared to reflect the rate of erythropoiesis since it decreased in aplastic anemia and increased in hemolytic anemia.[4] This report further characterizes the human plasma receptor material and describes an enzyme-linked immunosorbent assay (ELISA) to quantitate transferrin receptor levels in human plasma. Results of this measurement in a variety of red blood cell (RBC) disorders in humans are presented. In addition, a comparison was made between plasma transferrin receptors and ferrokinetic measurements of iron-bearing uptake by erythroid receptors, which is known to be closely related to the rate of erythropoiesis.[5]

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MATERIALS AND METHODS

Procedure for plasma receptor characterization. Human placental transferrin receptors, purified in the form of a receptor-transferrin complex as described elsewhere,[6] were used in the characterization of plasma receptor material. Iodination of the placental complex was performed by the method of McFarlane,[7] with ^{125}I as sodium iodide (specific activity 13 to 17 Ci/μg iodine). Radioiodinated transferrin in the complex was removed by exchange with an excess of cold human diferric transferrin, followed by electrophoretic separation of transferrin from receptor-transferrin complexes.

A trace amount of radioiodinated placental receptor-transferrin complex was added to ^{59}Fe-labeled plasma. Electrophoresis of 0.25 mL of this doubly labeled plasma was performed in an electrophoretic cell model 155 (Bio-Rad, Richmond, CA), using 6% polyacrylamide gels in a column of 1.4 × 12 cm. Plasma was subjected to electrophoresis for 16 hours at 80 V and 2 hours at 160 V. Then the gel was cut in slices of 2 mm. The content of each slice was eluted for 48 hours in 1 mL 0.9% saline containing 0.4% TERIC, and analyzed ^{125}I were counted in all the fractions, as described above.

Transferrin antibodies, prepared as described elsewhere,[8] were added to ^{59}Fe-labeled plasma samples and incubated for 2 hours at 37°C to precipitate transferrin. After centrifugation for 20 minutes at 2,000 × g, the plasma supernate was analyzed for receptor content by ELISA, and ^{59}Fe was counted in both pellet and supernatant.

Production of transferrin receptor antibodies. Rabbits were immunized by repeated weekly injections of 100 μg of receptor-transferrin complex and bled to obtain serum after 2 to 3 months. The immunoglobulin G (IgG) fraction of rabbit serum was isolated by ammonium sulfate precipitation and gel chromatography using a double column method.[9] The purified IgG fraction was then passed through a column (1 × 5 cm) of human diferric transferrin coupled to Affigel (Bio-Rad, Richmond, CA) 10 or 15 (10 mg/5 mL gel) to remove transferrin antibodies produced because of the transferrin present in the complex injected. The monospecific antibody was then dialyzed against 0.01 mol/L bicarbonated saline (pH 8.3) and stored at −28°C.

To demonstrate the specificity of the antibody for the receptor and its nonreactivity with transferrin, crossed immunoelectrophoresis[9]

was performed on pure transferrin and receptor-transferrin preparations, and pure transferrin preparations (5 to 10,000 μg/L) were analyzed by ELISA.

ELISA procedure. Microtiter plates containing 96 wells (Immunoplate I with certificate; Nunc Intermed, Roskilde, Denmark) were used by adding 250 μL of the absorbed IgG solution diluted 1:2,500 in 0.05 mol/L sodium carbonate buffer (pH 9.6) to each well. Plates were incubated for 1 to 14 days at 4°C before use. On the day of the assay, the coating solution was removed and the unreacted sites were blocked by adding 250 μL of 0.5% bovine serum albumin (BSA) (Sigma, St Louis, MO) in 0.05 mol/L sodium carbonate buffer, pH 9.6. After 30 minutes incubation at room temperature (RT), each plate was emptied and washed three times with at least 300 μL 0.15 mol/L phosphate-buffered saline (PBS) containing 0.05% Tween 20, allowing each wash to stand for at least 3 minutes at RT. Plates were emptied by sharply inverting them and dried by tapping on paper towels and vacuum aspiration.

Two hundred microliters of blanks, diluted plasmas, and standards were added within 30 minutes, using an automatic pipetor (Micromedic System, Inc, Philadelphia, PA). Unknown plasmas were diluted 1:200 to 1:2,000 with 0.15 mol/L PBS (pH 7.4) containing 0.5% BSA and 0.05% Tween 20. Two successive dilutions of the tested plasma were usually necessary. The stock standard solution (2 μg receptor-transferrin complex/mL) contained 0.4% TERIC and was further diluted with the blank solution for the assay. The plates were covered with parafilm and incubated either 2 hours at RT or overnight at 4°C. The wells were then emptied and washed as described above.

Horseradish peroxidase was conjugated to anti-receptor IgG as described elsewhere.[10] Two hundred microliters of this conjugate diluted 1:2,500 in 0.15 mol/L PBS containing 1% BSA and 0.05% Tween 20, were added to each well. Plates were covered with parafilm and incubated for 2 hours at RT. The washing step was then repeated.

Two hundred microliters of freshly prepared substrate were added to each well. The substrate consisted of 340 mg/L o-phenylenediamine (Sigma) in freshly mixed 0.15 mol/L citrate phosphate buffer (pH 5.0), with 0.01% hydrogen peroxide. Plates were covered with parafilm and incubated for 30 minutes in the dark at RT. The reaction was terminated by adding 50 μL of 12.5% sulfuric acid to each well. Absorbance was read at 492 nm within 30 minutes using a plate reader (Bio-Tek Instruments, Inc, Burlington, VT), after setting the reader to zero with 0.15 mol/L PBS containing 0.05% Tween. The reagent blank was subtracted from each value and the receptor-complex level in the unknown plasma was derived after plotting its absorbance against the standard curve. The protein content of the standard transferrin receptor complex was determined by the Bio-Rad protein assay and by measuring the absorbance at 280 nm, with an absorbance of 1 corresponding to 1.06 mg protein/mL.[11] It was essential that different areas of the plate be monitored by standards, because some plates gave unsatisfactory readings at the periphery. Each sample was run in triplicate at two different dilutions, and the mean value presented for each sample in micrograms per liter plasma. Results were also expressed in micrograms per liter whole blood.

Erythron transferrin uptake. Ferrokinetic measurements involved the initial calculation of plasma iron turnover from the plasma iron concentration, the disappearance rate of radioiron from the plasma, and the plasmatocrit.[12] Extravascular flux, a function of plasma iron concentration,[13] was then subtracted. The effect of iron concentration was removed by converting tissue iron uptake to iron-bearing transferrin uptake, and nonerythron uptake was subtracted.[14] Erythron transferrin uptake (ETU) was expressed as micromoles per liter whole blood per day. Results were also corrected for abnormalities in blood volume by the ratio of measured (from the dilution of the radioiron injected) to predicted blood volume.[15]

Patients. Heparinized blood samples were obtained, and plasma was separated within 1 hour and stored frozen at −28°C until processing. A total of 316 subjects were studied. These included 56 normal subjects as determined by standard hematologic screening including serum ferritin, 32 men and 24 women, aged 18 to 78 years. There were 43 patients with chronic renal failure, 16 with aplastic anemia at the time complete aplasia can be assumed, and 11 who had been treated by bone marrow transplantation. There were 16 patients with hemolytic anemia: 7 with hereditary spherocytosis and 9 with autoimmune hemolytic anemia. Eighty-three patients with thalassemia included 72 with β-thalassemia/hemoglobin E and 11 with hemoglobin H disease. Thirteen patients had iron deficiency without anemia and seven had idiopathic hemochromatosis treated by phlebotomy over several years. There were 12 patients with polycythemia vera, 4 with chronic and 3 with acute myelogenous leukemia, 10 with myelofibrosis, 10 with a myelodysplastic syndrome, and 5 with essential thrombocythemia. Twenty-three patients had a nonmyeloid malignancy, either a solid tumor (nine patients with lung, breast, or ovarian cancer) or a lymphoid malignancy (14 patients with lymphoma, chronic lymphocytic leukemia, or multiple myeloma) with little or no marrow dysfunction. Ferrokinetic studies were performed on 148 of these subjects. Informed consent was obtained according to a protocol approved by the University of Washington Human Subjects Committe (Seattle).

Miscellaneous. Hematocrit was determined by the micromethod technique or with a Coulter S Counter (Marietta, GA). Plasma iron and total iron-binding capacity were determined by standard methods.[16,17] Plasma ferritin was measured by a standard radioimmunoassay.[18]

RESULTS

Characterization of plasma receptors. Three plasmas with receptor-complex determinations by ELISA of 8,221, 12,252, and 46,835 μg/L were incubated with transferrin antibodies and the precipitate removed by centrifugation. Receptors, 95% ± 3%, as determined by ELISA, had disappeared from the supernate. Thus, removal of transferrin by specific precipitating antibodies also removed virtually all receptors from the plasma.

After gel filtration through the AcA34 column, the receptor peak detected by ELISA again coincided with the radioiodinated placental receptor peak (Fig 1A). Thus, circulating receptors appeared to be in the same molecular form as placental receptors.

After electrophoresis of ^{59}Fe-labeled plasma samples containing a trace amount of ^{125}I-Labeled placental receptor-transferrin complex, there was a single peak (centered on fraction no. 19) of receptor activity detected by ELISA in virtually the same position as the radioiodinated tissue receptor (centered on fraction no. 17), well behind the ^{59}Fe transferrin peak (centered on fraction no. 38) (Fig 1B).

Assay characteristics. The specificity of the receptor antibody was documented by the absence of reaction with transferrin in crossed immunoelectrophoresis as well as in the ELISA assay. Crossed immunoelectrophoresis of receptor-transferrin complexes showed a single peak.

Sensitivity, as defined by the lowest measured receptor complex concentration that differed significantly from background ($P < .001$), was at least 0.725 μg/L. The dose response curve was linear in the 0.725 to 29 μg/L range, then

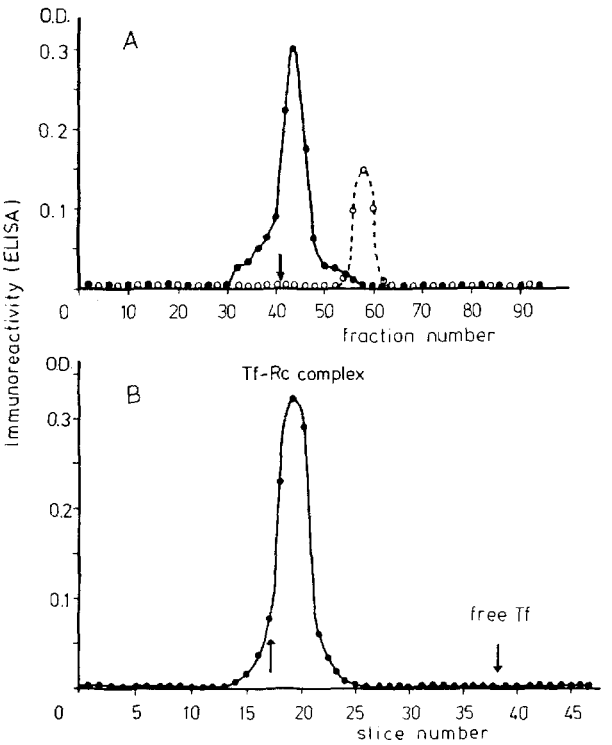

Fig 1. Detection of TfRc complex in human plasma by immunoreactivity (ELISA technique). (A) Separation by gel permeation chromatography on LKB AcA 34 (5 × 95 cm; fraction volume 18.5 mL, flow rate: 2 mL/min; buffer: 0.05 mol/L Tris/HCL, pH 8.3, containing 0.15 mol/L NaCl and 0.5% TERIC.[11] Volume of normal human plasma applied: 20 mL. Arrows indicate the position of the intact, ^{125}I-tagged human placental receptor. The broken line represents the elution pattern of ^{125}I-tagged human transferrin. (B) Separation on PAGGE electrophoresis in 5% polyacrylamide.[11] Electrophoretic separation was performed in 1.5 × 12.5 cm gel columns in a BioRad Model 155 electrophoretic cell for 24 hours at 80 V and 40°C.[11]

slightly curvilinear in the 29 to 58 μg/L range. The maximum absorbance reading of 2.0 was usually attained beyond the 58 μg/L value. No inhibition of the dose response by excess antigen was observed with concentrations up to 1,000 μg/L.

The within-assay variability was between 3% and 4% (coefficient of variation) when eight replicates were performed on plasmas with receptor concentration between 7.25 and 58 μg/L, and 4.3% when the same sample was run in 72 wells. The between-assay variability was evaluated by measuring the same two control plasmas in each plate over a 6-month period. The coefficients of variation were 7.6% and 8.1%, respectively. Reproducibility assays by two different investigators (PP and YB) gave consistent results and their coefficients of variation were similar.

Receptor content of plasma samples in hematologic disorders. Plasma receptor levels in normal subjects and patients with various conditions are presented in Table 1. The mean value in 56 normal individuals was 8,279 ± 1,261 μg/L. There was no significant difference between males (8,395 ± 1,203 μg/L) and females (8,076 ± 1,247 μg/L), nor between subjects under (8,511 ± 1,116 μg/L) or over 35 years of age (7,902 ± 1,319 μg/L).

In patients with hypoproliferative anemia, receptor levels were decreased. In aplastic anemia, levels averaged 47% of normal. It was recognized that a number of patients with presumed aplasia might have spotty marrow activity, not detected by marrow examination. A better approximation of true aplasia might be derived from the five patients with the lowest values whose mean was 2,566 μg/L or about one third that of the normal value. In patients with severe renal failure, receptors averaged 60% of normal. Erythroid hypoplasia is also found in patients exposed to marrow-suppressive drugs before bone marrow transplantation. Their receptor levels averaged 46% of normal. Their levels were lower in the early posttransplant period (2,175 to 4,248 μg/L, between days 15 and 150) but almost normal (5,017 to 6,017 μg/L) after 180 days.

Receptor levels showed a mean increase of 3× normal in patients with autoimmune hemolytic anemia and 4.5× in hereditary spherocytosis. While values in hemoglobin H disease were increased only three times, the more anemic subjects with β-thalassemia/hemoglobin E had a mean increase of 8.5 × normal.

Patients with iron deficiency presented a wide range of values (from 11,237 to 80,707 μg/L). The highest values of 68,657, 69,266, and 80,707 μg/L were seen in three subjects with hematocrits of less than 15. Receptor levels were within the normal range in patients with idiopathic hemochromatosis.

Polycythemia vera patients and those with myelofibrosis had a mean value 2 to 3× normal and ranged from normal to 6× normal. The three highest values of 28,507, 32,523, and 51,794 μg/L were in patients with polycythemia vera who had associated iron deficiency. Patients with a myelodysplastic syndrome showed low to moderately increased values. Patients with chronic or acute myelogenous leukemia, essential thrombocythemia, as well as those with a lymphoid malignancy or a solid tumor, had essentially normal levels.

Relationship between receptor levels and ETU. One hundred forty-eight patients had ferrokinetic studies performed and their receptor levels determined on the same day. There was an excellent correlation between the ETU and plasma receptors ($r = .086$, $P = .0000$) (Fig 2). When circulating receptor levels were expressed as micrograms per liter whole blood, the correlation coefficient with the ETU remained basically unchanged ($r = .82$, $P = .0000$). In a multivariate correlation analysis with plasma iron, total iron binding capacity (TIBC), ferritin, hematocrit (Hct), and ETU as independent variables, the latter was the best single determinant for receptor levels ($r = .88$), ie, the multiple r correlation coefficient only increased to .94 with the other variables. In the different groups of subjects, the relationship between plasma receptors and erythroid receptors as evaluated by the ETU appeared unaffected by the particular disease state studied (Table 2).

DISCUSSION

Membrane transferrin receptors are essential to supply transferrin iron to body tissues.[1] The total number of receptors determines the amount of iron uptake, and the varying numbers of receptors in different tissues account for

Table 1. Plasma Transferrin Receptors in Subjects With a Variety of Hematologic Disorders

	N	Age (yrs)	Hct (%)	Receptors* (μg/L)	Range	P Value
Normal subjects	56	37 ± 16	43 ± 6	8,279 ± 1,261	5,307-11,063	—
Hypoplastic erythropoiesis						
Aplastic anemia	16	35 ± 16	16 ± 5	3,871 ± 1,145	2,175-5,930	.0000
Marrow transplantation	14	30 ± 10	32 ± 5	3,842 ± 1,348	2,073-6,017	.0000
Chronic renal failure	43	45 ± 14	21 ± 3	4,973 ± 2,276	1,044-10,205	.0000
Hyperplastic erythropoiesis						
Immune hemolytic anemia	9	50 ± 13	35 ± 5	23,258 ± 5,640	14,558-31,117	.0000
Hereditary spherocytosis	7	31 ± 19	33 ± 4	37,076 ± 12,847	16,457-51,794	.0001
β-thalassemia/Hb E	72	26 ± 8	22 ± 4	70,049 ± 22,417	29,710-142,941	.0000
Hemoglobin H disease	11	40 ± 13	35 ± 3	32,900 ± 8,352	22,083-48,386	.0000
Altered iron status						
Iron deficiency	13	47 ± 16	32 ± 15	360,325 ± 23,490	11,237-80,707	.0000
Idiopathic hemochromatosis	7	61 ± 9	41 ± 5	9,497 ± 3,712	5,321-16,617	NS
Malignancies						
Polycythemia vera	13	57 ± 16	54 ± 8	22,011 ± 11,846	10,715-51,794	.0000
Myelofibrosis	10	70 ± 9	29 ± 6	12,615 ± 3,103	8,076-18,067	.0000
Myelodysplastic syndrome	10	57 ± 19	31 ± 10	12,310 ± 5,582	4,814-22,083	NS
Acute myelogenous leukemia	3	53 ± 8	35 ± 4	8,352 ± 3,364	5,495-12,035	NS
Chronic myelogenous leukemia	4	64 ± 9	38 ± 3	8,192 ± 2,131	5,307-10,382	NS
Essential thrombocythemia	5	47 ± 6	41 ± 2	7,525 ± 957	6,278-8,830	NS
Lymphoid malignancies	14	67 ± 10	41 ± 7	9,497 ± 3,190	6,343-17,425	NS
Solid tumors	9	63 ± 7	44 ± 2	7,859 ± 1,450	5,858-10,034	NS

Values as mean ± SD.

*Values refer to the transferrin receptor molecule complexed with transferrin.

the marked differences in uptake by those tissues. In normal humans, more than 80% of essential body iron ultimately is used for erythropoiesis,[10] and a proportionate number of transferrin receptors appear to reside in erythroid marrow. These receptors and the capacity to take up iron are lost as the cell matures through the reticulocyte stage.[19]

The discovery of material in human plasma that reacts with receptor antibodies[3] might be expected from the demonstration of receptor exocytosis in cell culture.[20] In this report, the reacting material has been shown to be a complex of transferrin and transferrin receptors. No evidence of receptor fragments was found in the plasma by either electrophoresis or gel filtration. Behavior of this complex was similar to that of isolated placental complexes, which have been assumed to contain receptor and transferrin in a 1:1 molar ratio.[6,11]

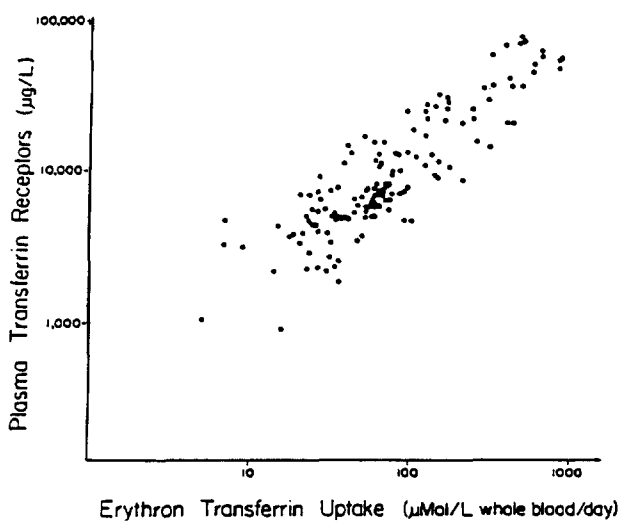

Fig 2. Correlation between levels of plasma transferrin receptor molecules complexed with transferrin and ferrokinetic measurements of ETU in 148 patients ($r = .86$, $P = .0000$).

The amount of plasma receptors was estimated by an enzyme-linked polyclonal antibody assay, using purified placental receptor-transferrin complexes as a reference standard. The similar nature of plasma and placental receptors validated the use of placental receptor-transferrin complexes as a standard for the ELISA assay, if allowance was made for the transferrin present in the complexes. Mean value for normal subjects found by our assay was 8,279 μg/L, compared with the value of 367 μg/L reported by Kohgo et al.[3] The more than 20-fold difference was most likely a result of the greater affinity of our polyclonal antibody compared with their monoclonal antibody.

The finding of Kohgo et al[4] that receptor levels were increased in hemolytic anemia and decreased in aplastic anemia suggested that a constant relationship might exist between membrane receptors and plasma receptors. If so, the receptor assay could be useful in monitoring erythropoiesis.

The present clinical observations provide more quantitative information about the role of the erythron as a determinant of the plasma receptor level. In severe aplastic anemia or severe renal failure, levels were reduced to about one third of the basal level. This demonstrates the dominant role of the erythron, but also identifies the significant fraction contributed by other tissues. Receptor levels in congenital hemolytic anemia increased as much as six times above basal levels, and levels greater than 10 times above basal levels were seen in some patients with thalassemia. The very high receptor levels

Table 2. Comparison of Plasma Transferrin Receptors and ETU

Diagnosis	N	Hct (%)	ETU (μmol/L)	Receptors* (μg/L plasma)
Normal subjects	17	47 ± 5	60 ± 13	7,772 ± 1,029
Chronic renal failure	43	21 ± 3	33 ± 15	4,973 ± 2,276
Hemolytic anemia	11	33 ± 4	313 ± 135	32,639 ± 12,412
Hemoglobin H disease	11	35 ± 3	229 ± 102	32,900 ± 8,352
β-thalassemia/hemoglobin E	11	24 ± 5	562 ± 206	67,903 ± 17,008
Polycythemia	13	52 ± 5	114 ± 46	17,429 ± 8,346
Myelofibrosis	10	29 ± 6	158 ± 66	12,615 ± 3,103
Myeloid disorders†	12	38 ± 4	69 ± 46	7,975 ± 2,218

Mean ± 1 SD.

*Myelodysplastic syndrome, chronic myelogenous leukemia, essential thrombocythemia.

†Values refer to the transferrin receptor molecule complexed with transferrin.

in thalassemia patients indicate that receptors are derived not only from the maturing reticulocyte,[20] but also from abnormal immature erythroid cells destroyed at an earlier stage. Receptor levels that we obtained from patients with a variety of other conditions appeared to be consistent with our understanding of erythropoiesis in these conditions.

Another measurement also reflects the functional activity of transferrin receptors. The ferrokinetic measurement of ETU measures the number of iron-bearing transferrin molecules taken up by tissue receptors per unit time.[14] The close relationship between ETU and the receptor measurement (Fig 2) reflects the parallel changes in the number of immature erythroid cells that occur in various hematologic disorders. If these two measurements agree, the receptor complement of individual cells must be essentially the same.

The relationship of the transferrin receptor number to erythropoiesis exists only when there is sufficient iron-bearing transferrin to saturate receptors. When iron deficiency exists, both erythroid and nonerythroid receptors increase,[1,21] and the relationship between receptor number and erythropoiesis is distorted. The ferrokinetic measurements in iron-deficient patients are also no longer valid as a measure of erythroid marrow function, because iron supply rather than erythroid cellularity determines the ETU. Inflammation may also have an additonal effect on plasma receptor levels as shown in the rat.[11] Although malignant nonerythroid cells have been shown to have increased numbers of transferrin receptors,[1] this increase does not appear to have a significant effect on total plasma receptor levels (Table 1).

Finally, these studies illustrate the potential utility of trace plasma protein measurements as an indicator of the behavior of cell proteins. In the present instance, cells of a particular type (erythroid precursor) possess most of the body's transferrin receptors. Therefore, the size of that tissue may be assessed from the plasma receptor level. The measurement of plasma levels of glycocalicin, a fragment of a platelet-membrane protein, has proved valuable recently in the assessment of megakaryopoiesis.[22] The plasma ferritin assay has demonstrated its value by indicating the intracellular amount of that protein and its iron stores.[23] Plasma assays for other membrane or intracellular proteins that show selective tissue localization may also be worth undertaking.

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