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Quantitation of Apo-, Mono-, and Diferric Transferrin by Polyacrylamide Gradient Gel Electrophoresis in Patients With Disorders of Iron Metabolism

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We have developed a polyacrylamide gradient gel electrophoretic method to quantitate apo-, mono-, and diferric transferrin based upon differences in their molecular size. Purified transferrin saturated to different extents (3% to 98%) with iron showed proportions of the three forms as predicted from an approximately random distribution of iron between the two metal-binding sites. The iron distributions in sera of 14 normal individuals similarly correlated with the predicted values. In contrast, 22 of 43 patients with diseases associated with abnormalities in iron or transferrin metabolism had a disproportionate increase in monoferric transferrin. This abnormality occurred in seven of nine patients who had received bone marrow transplants, seven of 14 with chronic liver disease, and eight of nine menstruating women with probable iron deficiency anemia. Interestingly, 11 patients with malabsorption or chronic renal disease had normal iron distributions. The finding of abnormal distributions of iron on transferrin suggests that gradient gel analysis may be a useful tool for studying the physiologic mechanisms controlling iron utilization.

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TRANSFERRIN is the major iron-binding protein in serum. It is necessary for lymphocyte proliferation,[1] hematopoiesis,[2] and other physiologic processes.[3] The apo-, mono-, and diferric forms of transferrin can be distinguished by differences in susceptibility to denaturation by urea,[4] in charge as measured by isoelectric focusing,[5] in absorbance at 470 nm,[6] and in their size.[7]

Studies of the distribution of iron on transferrin in normal human serum are conflicting. Huebers et al[8] recently concluded that iron distributes randomly between the two metal-binding sites of transferrin in normal individuals. Earlier studies by Leibman and Aisen[9] and by Williams and Moreton[10] suggested that the *N*-terminal site is preferentially occupied.

The distribution of iron on transferrin in patients with diseases in which abnormal iron metabolism might occur have not been reported. These include patients with malnutrition or chronic inflammation in which the serum transferrin levels may be decreased due to underproduction by the liver,[11] those with the nephrotic syndrome where transferrin levels may be low due to urinary loss,[12] and those who have had bone marrow transplants.[13] In this study, we developed a procedure for quantitating the relative proportions of apo-, mono-, and diferric transferrin in serum using polyacrylamide gradient gel electrophoresis (PAGGE) which separates nondenatured proteins by differences in molecular size.[14] This technique was used to analyze the distribution of iron among the forms of transferrin in normal individuals and in several groups of patients with diseases associated with disorders of iron metabolism.

MATERIALS AND METHODS

Patient and normal populations. Human serum samples were obtained from normal volunteer donors or from patients at Emory University Hospital using protocols approved by the Emory University Human Investigation Committee. Sera were obtained from 14 normal individuals (13 men and one woman) and nine otherwise healthy women who were presumed to have at least mild iron deficiency (the percentage of saturation of their serum transferrin was 30%). Clinical samples were obtained from nine patients who had received bone marrow transplants (one autologous and eight allogeneic) one to four weeks previously. Of these, 3 had acute myelogenous leukemia, 2 had acute lymphocytic leukemia, 1 had chronic granulocytic leukemia, 1 had chronic lymphocytic leukemia, and 2 had aplastic anemia. Sera were also obtained from 14 patients with chronic liver disease (13 with alcoholic cirrhosis and one with portal vein thrombosis), six malnourished patients undergoing intravenous hyperalimentation therapy for malabsorption syndrome, and five patients with renal disease (three with minimal lesion nephrotic syndrome, one with isolated familial proteinuria, and one with diffuse membranoproliferative glomerulonephritis). Sera not immediately assayed were stored frozen at −80 °C.

Preparation and measurement of iron transferrin. Human serum transferrin (tissue grade, 99% iron free) was obtained from Sigma Chemical Co (St Louis). Iron-loaded transferrin was prepared by adding varying amounts of iron–nitrilotriacetate in a 1:2 ratio to apo-transferrin in phosphate-buffered saline, pH 7.6, yielding percent saturations ranging from 3% to 96%. This procedure results in a preferential binding of iron to the *C*-terminal site of transferrin.[9] To insure that the anion binding sites of transferrin were not occupied by nitrilotriacetate, a 50-fold excess of bicarbonate was added to each sample.[9]

Transferrin concentration was measured immunochemically using an ICS Analyzer II kinetic nephelometer (Beckman Immunochemistry Systems, Fullerton, Calif). Serum iron was measured using a ferrozine assay adapted in our laboratory to the COBAS-BIO Centrifugal Analyzer (Roche Analytical Instruments, Nutley, NJ).[15] The percentage of saturation of transferrin was calculated as

$$\frac{\text{Serum iron } (\mu g/dL)}{\text{Total iron-binding capacity } (\mu g/dL)} \times 100.$$

In normal individuals in our laboratory, the relationship between serum total iron-binding capacity measured in $\mu g/dL$ is equivalent by regression analysis to the serum transferrin measured in mg/dL. Because serum transferrin values give a more accurate assessment of total high-affinity iron binding in serum in diverse patient populations than do measurements of total iron-binding capacity,[16,17] serum transferrin values were used in all calculations of the percentage of saturation. Saturation of purifed transferrin was also calculated by measuring the absorbance of iron transferrin at 470 nm[6] as follows. The absorbance values of duplicate samples of transferrin (~1.3 mg/mL) treated with excess iron and assumed to represent 100% saturated transferrin were measured; the spectrophotometer was blanked against buffer and the 0 value was taken to represent

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unsaturated transferrin. The absorbance values of identical concentrations of transferrin samples treated with various intermediate amounts of iron were measured and the percentage of saturation of each sample was calculated from Beers' law, assuming a direct relationship between absorbance and the percentage of saturation.

Electrophoretic methods. PAGGE was done using 2.5% to 27% concave gradient gels (Isolab, Akron, Ohio). Two gels, each with six samples, could be run concurrently. Gels were electrophoresed in a Tris–borate–EDTA buffer (0.01 mol/L Tris, 0.0026 mol/L EDTA, 0.08 mol/L borate, pH 8.4) for 18 to 22 hours at a constant voltage of 100 V. Immediately after electrophoresis, the gels were fixed in 0.7 mol/L trichloroacetic acid and 0.14 mol/L 5-sulfosalicyclic acid, then stained with Coomassie blue.

The transferrin bands were identified immunochemically by the Western blot technique.[18] After electrophoresis, the gels were sliced into two 1.5-mm-thick sections. One was stained with Coomassie blue, the other was vacuum-blotted onto nitrocellulose paper and reacted overnight with goat anti-human transferrin serum (1:500; Atlantic Antibody, Scarborough, Me). The paper was rinsed with 0.05% gelatin in Tris buffer (0.05 mol/L, pH 7.4), then reacted with peroxidase-conjugated rabbit anti-goat serum (1:100 dilution, Cappel Labs, Cochranville, Pa) for one hour at room temperature and rinsed again in gelatin buffer. The transferrin bands were visualized by reaction with 3,3'-diaminobenzidine tetrahydrochloride in hydrogen peroxide (Polysciences Inc, Warrington, Pa).

Since the transferrin bands were spaced too closely to be scanned directly by conventional densitometry, we enlarged 35-mm negatives (Technical Pan 35 mm, Kodak, Rochester, NY) of the gels onto 4 × 5-inch negative sheet film (Technical Pan). The resultant positive image (enlarged approximately four to five times) was then scanned using a Cliniscan densitometer (Helena Laboratories, Beaumont, Tex). We have compared data obtained in this way with results obtained by direct scanning of the gels using a laser densitometer (LKB Instruments, Gaithersburg, Md) and the two methods gave comparable results.

Urea gel electrophoresis was performed by the methods of Makey and Seal.[19] Briefly, 6% acrylamide gels were made using 6.3 mol/L ultrapure urea (Schwarz/Mann, Cambridge, Mass) in a 0.01 mol/L Tris buffer at pH 8.6. Purified transferrin samples were diluted 1:2 with 6% sucrose. A total of 6 to 10 μg of sample in 10 to 50 μL was applied to the gels, which were then electrophoresed for six to eight hours in the same Tris–borate–EDTA buffer as that used in the gradient gel electrophoresis. The bands on the urea gels were quantitated by densitometry.

RESULTS

Identification and quantitation of apo-, mono-, and diferric transferrin on polyacrylamide gradient gels: Separation of iron forms in purified transferrin. We prepared a series of transferrin samples with a range of iron saturation from 3% to 98%. The percentage of saturation was verified by iron and transferrin measurements, and by absorbance at 470 nm. The samples were electrophoresed on polyacrylamide gradient and urea polyacrylamide gels (Fig 1). One to three bands were observed on the gradient gels and one to four on urea gels. The ratio of the bands changed with iron content. At low percentage saturations, the slow migrating transferrin band (apo-transferrin) predominated. At high percentage saturations, the fast migrating transferrin band (diferric transferrin) predominated. By adding graded amounts of iron, we determined that the middle band on the gradient gels was monoferric transferrin. The two middle bands on urea gels have previously been shown to represent

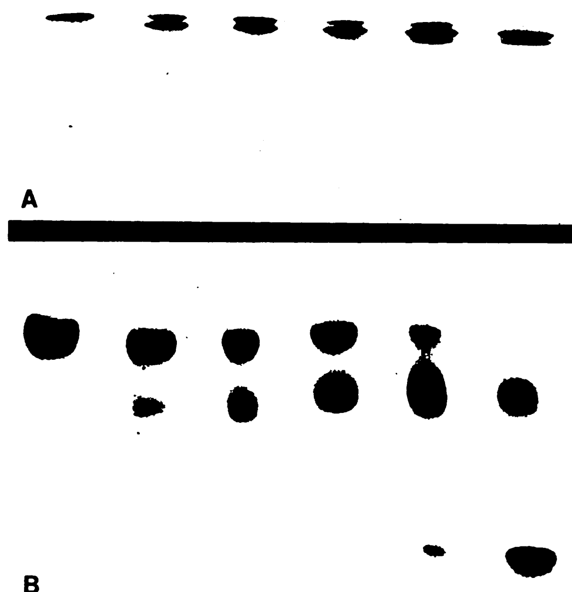

Fig 1. Gradient gel (A), and urea gel electrophoresis (B) patterns of a sample of purified transferrin sample saturated with iron (iron–nitrilotriacetate) to yield percentages of saturation of 3%, 18%, 28%, 35%, 56%, and 75%.

the *N* and *C* terminal iron forms of monoferric transferrin.[4] The percentage of saturation of iron was calculated from densitometric quantitation of the transferrin bands on the gels using the following formula:

$$\% \text{ Saturation} = (\% \text{ monoferric transferrin}/2) + (\% \text{ diferric transferrin}).$$

The following experiments were performed to determine how these methods of measuring the percentage of transferrin saturation correlated with one another. Figure 2 shows the relationships between the percentage of saturation of purified transferrin as calculated from serum iron and transferrin measurements and that calculated using gradient gel electrophoresis (r = .97), urea gel electrophoresis (r = .95), and iron transferrin absorption (r = .95). The urea gel consistently showed less diferric transferrin than did the other methods. This was probably due to the EDTA in the buffer, as EDTA can remove iron from transferrin.[20] When we performed urea gel electrophoresis at a higher pH (pH 8.8) and without EDTA, the proportion of diferric transferrin was appropriately increased.

In order to determine whether gradient gels could also be used to quantitate apo-, mono-, and diferric transferrin, we compared them with urea gels. There was good agreement between the percentages of monoferric transferrin obtained from both methods (r = .91), and these correlated with the expected values calculated from the percentages of saturation (r = .90) (Fig 3). The observed and expected proportions of apo- and diferric transferrin were also comparable to those

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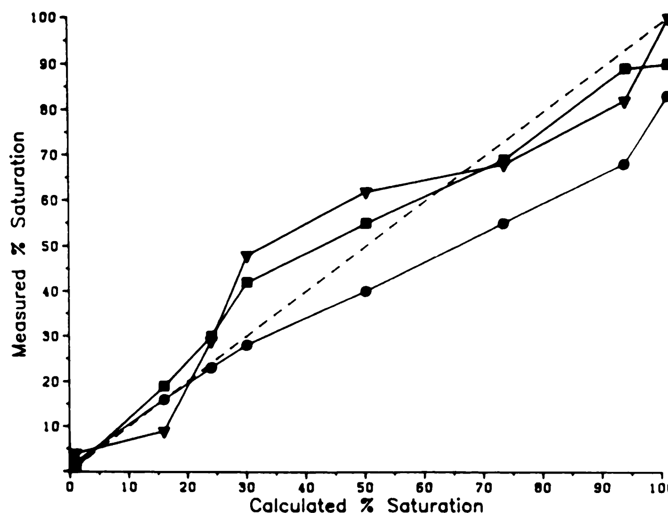

Fig 2. Correlation of different methods for measuring the percentage of saturation of transferrin with iron. Purified transferrin was saturated with increasing amounts of iron–nitrilotriacetate. The percentage of saturation as calculated from iron and transferrin measurements is plotted against the percentage of saturation obtained from gradient gels (■), urea gels (●), and absorption of iron transferrin at 470 nm (▼).

calculated from iron and transferrin measurements (apotransferrin, $r = .95$; diferric transferrin, $r = .96$).

Reproducibility of gradient gel separation of the three iron forms of transferrin was studied by running a single sample 16 times in parallel with serum samples over a six-month period. The specimen was stored frozen at −20 °C in a single aliquot, and it was thawed and refrozen many times without affecting the distribution of the transferrin bands. The coefficient of variation was <11% for each band (the percentage of apotransferrin [mean ± SD], 33 ± 3.1; the percentage of monoferric transferrin, 44 ± 4.2; and the percentage of diferric transferrin, 24 ± 2.6).

Distribution of iron among apo-, mono-, and diferric transferrin in normal and patient sera. All of the bands observed using purified transferrin were also identified in serum samples (Fig 4). In serial samples from a single patient undergoing central venous hyperalimentation therapy for malabsorption syndrome, the gradient gels showed changes in both the concentration of transferrin and its percentage of saturation. The values were low initially, but rapidly returned toward normal during therapy (Fig 5). The percentages of saturation calculated by densitometric scanning of the transferrin bands closely paralleled those determined from iron and transferrin measurements.

We measured iron and transferrin concentrations in sera from 14 normal individuals to determine the percentages of saturation, and calculated the relative amounts of apo-, mono-, and diferric transferrin expected if the iron distributed randomly on transferrin.[21] The correlation between the percentage of saturation calculated from iron and transferrin measurements and that obtained from the gradient gels was very good ($r = .98$). The distribution of the iron forms also correlated well with the predicted distribution (Fig 6A) (apo-transferrin, $r = .95$; monoferric transferrin, $r = .90$; and diferric transferrin, $r = .96$).

We performed similar studies on 43 patients with diseases associated with abnormal iron metabolism. Seven of nine patients who had undergone bone marrow transplants showed 14% to 31% more monoferric transferrin on gradient gels than seen with normal individuals with comparable percentages of saturation (Fig 6B). Two of the patients with increased monoferric transferrin had less diferric transferrin than predicted; the others had less apotransferrin.

To determine if there might be a defect in the binding of

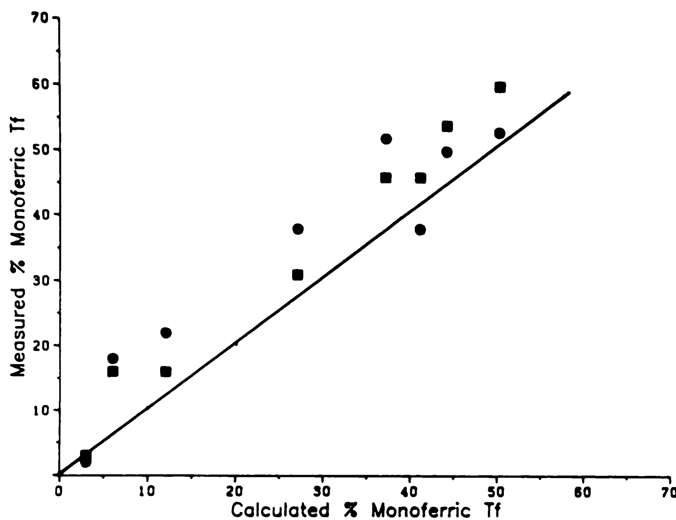

Fig 3. Comparison of the percentage of monoferric transferrin as measured by gradient (■) and urea (●) gel electrophoresis with the percentage of monoferric transferrin as calculated from iron and transferrin measurements. Transferrin samples saturated with increasing amounts of iron–nitrilotriacetate are the same as those used in Fig 2. The slight but consistent (5% to 10%) increases in monoferric transferrin above the values expected from a random distribution are just outside the experimental error of the densitometric quantitation of the bands on the gradient gels, but they are consistent with the previous observation that this method yields approximately fourfold differences in the binding of iron to the *N*- and *C*-terminal sites.[9]

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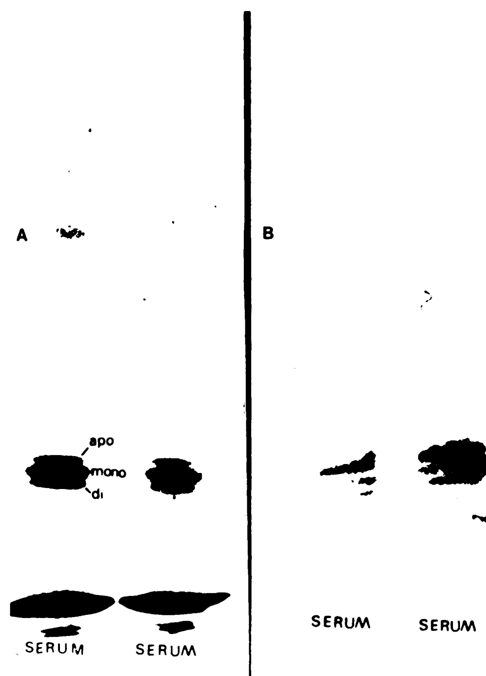

Fig 4. Gradient gel of a normal human serum sample run in duplicate. (A) The gel stained with Coomassie blue; (B) the immunoidentification of the three transferrin bands. The percentage of saturation of the serum sample was 58%.

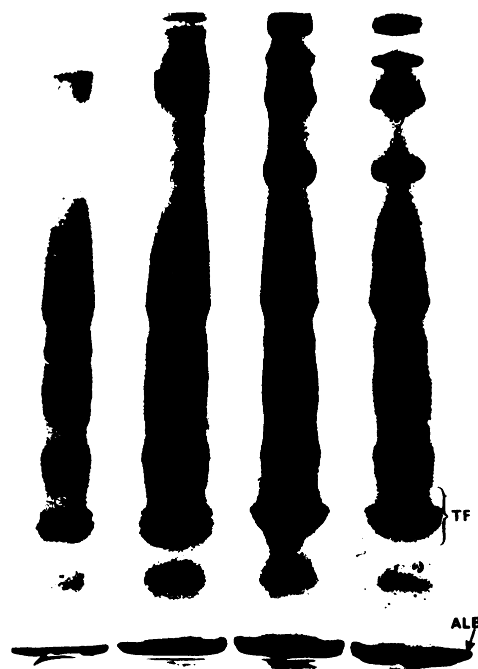

Fig 5. Gradient gel of serial serum samples of a single malnourished patient with Crohn's disease. From left to right, samples were drawn on days 5, 7, 12, and 17 after the start of central venous hyperalimentation. Transferrin values were 80, 108, 207, 220 mg/dL. Percentages of saturation were 23%, 66%, 11%, and 19%, respectively. TF, transferrin region; ALB, albumin region.

iron to transferrin, we investigated the iron-binding properties of the serum from one of the bone marrow transplant patients with elevated monoferric transferrin and decreased diferric transferrin percentages (observed values of apo-, mono-, and diferric transferrin [20%, 68%, and 12%] *v* predicted values [16%, 48%, and 36%] relative to its percentage of saturation (60%). The percentage of saturation calculated from the gradient gel was only 46%. When saturated with iron under acid conditions using excess ferric chloride, the procedure used for total iron-binding capacity measurements in our laboratory, and electrophoresed on a gradient gel, the patient's serum still showed only 76% saturation. A normal serum sample treated under the same conditions showed 97% saturation.

Seven of fourteen patients with cirrhosis of the liver and eight of nine normal menstruating women with presumed iron deficiency anemia also had increased proportions of monoferric transferrin (Table 1). All who had increased monoferric transferrin had decreased apotransferrin. All five patients with renal disease and six with malnutrition due to malabsorption had measured amounts of monoferric transferrin comparable to those of the normal persons.

DISCUSSION

The iron forms of serum transferrin were analyzed in normal individuals and in patients with suspected iron or transferrin abnormalities using a novel nondenaturing polyacrylamide gradient gel separation system. The gradient gels separated the iron forms of transferrin based on differences in their molecular size.[14] The principal advantage of this method is its relative simplicity and reproducibility. Unlike urea gels, which must be made just before use, the gradient gels are commercially available and are stable for several months. Since the transferrin bands migrate to a discrete region of the gradient gels, whole serum can readily be analyzed, whereas albumin and the alpha globulins must first be removed for urea gel analysis of the iron forms of transferrin.[22] The equilibrium electrophoretic technique of the gradient gels is also less susceptible to variations in temperature and timing of the run. The major disadvantage of the gradient gels is that they do not separate the *N* and *C* terminal monoferric forms of transferrin. For analysis of clinical specimens, however, PAGGE provided reproducible quantitation of the apo-, mono-, and diferric transferrin bands in the context of all other serum proteins.

In normal individuals, the distribution of iron on transferrin was as predicted by an approximately random distribution of iron between the two metal-binding sites, as calculated from serum iron and transferrin measurements, in agreement with the conclusions of Huebers et al.[8] Since the polyacrylamide gradient gel system cannot resolve the two monoferric forms of transferrin, we could not determine whether the iron bound preferentially to either site on transferrin. Huebers and co-workers [8,23] strongly suggest that the distribution of iron on transferrin is random, as shown using several methods including isoelectric focusing and cross-immunoelectrophoresis. The fact that these conclu-

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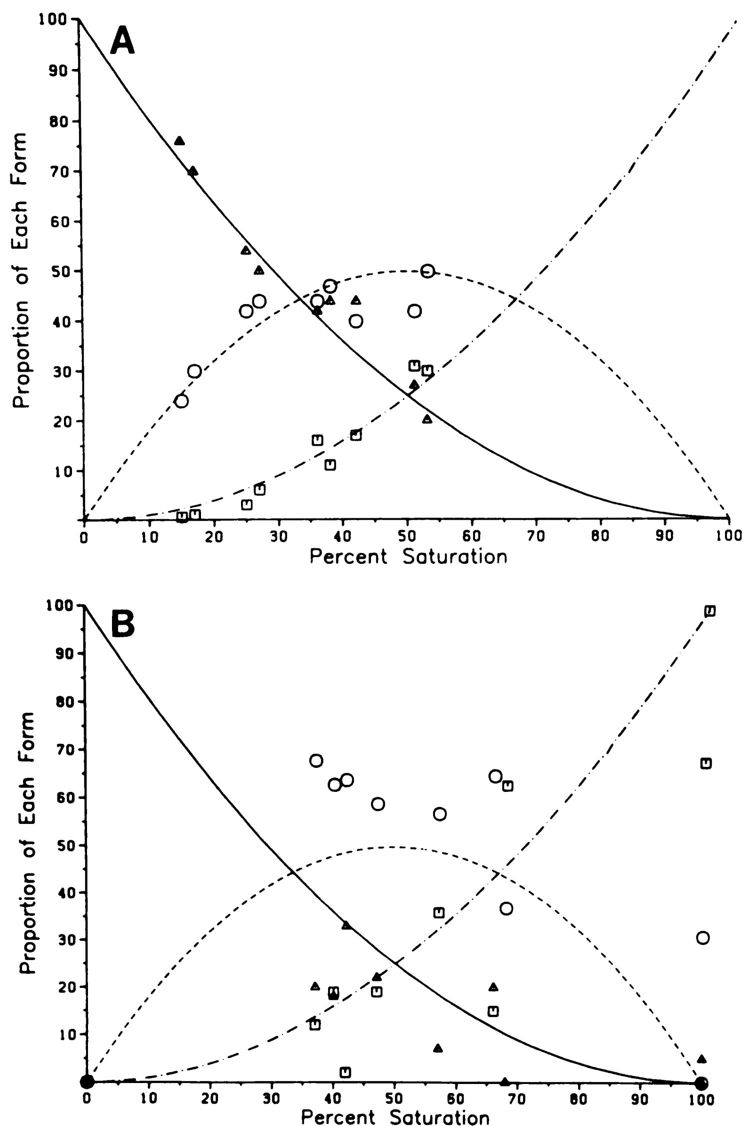

Fig 6. Proportion of apo- (▲), mono- (O), and diferric (⊡) transferrin in human sera as measured using gradient gels as a function of the percentage of saturation. (A) Results of 9 normal individuals; (B) results of nine bone marrow transplant patients. The curves represent the expected distribution of the transferrin forms as calculated from the equations of Chasteen and Williams,[21] assuming equal binding to *N*- and *C*-terminal sites on transferrin and no cooperativity between the sites.

sions contradict earlier studies[9,10] is attributed to a variety of technical factors that might have changed the native iron distribution. The close agreement between the patient values and the predicted distribution suggests that the analysis was not affected by possible iron contamination of the gradient gels or the buffer.

The patients formed two populations. The first group, consisting of severely malnourished patients and those with renal disease, had a distribution of iron on transferrin similar to that of the normal individuals. The second group, consisting of bone marrow transplant patients, patients with liver disease, and normal women who had a low percentage of saturation, had disproportionately elevated amounts of monoferric transferrin.

The increases in monoferric transferrin may reflect the fact that the steady state distribution is altered or may reflect the presence of defective molecules with altered iron-binding properties. In the first case, factors that alter the kinetics of binding, including those which affect iron loading or utilization rather than thermodynamic factors, may govern the distribution of iron in serum, while in the latter case, the equilibrium constants might differ from normal.

If the differences observed are due to alterations in iron loading or utilization, they do not simply reflect acute changes in iron concentration, since Huebers et al[8] showed that in normal individuals, short-term increases in iron loading, either from the intestine after oral ingestion of ferrous ascorbate or from the reticuloendothelial system after drug-induced hemolysis, do not alter the proportions of the two monoferric iron forms of transferrin.

Alternatively, if the altered iron transferrin distributions were due to changes in equilibrium constants, the magnitude of changes that would account for the data can be calculated using the equations derived by Chasteen and Williams.[21] We calculated the effects of changes in the iron-binding constants of transferrin on two parameters that we could measure using the gradient gels: the amount of monoferric transferrin at 50% iron saturation and the relative saturabil-

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Table 1. Difference Between Predicted and Observed Proportions of Monoferric Transferrin in Sera From Various Groups of Patients

	No. Tested	Serum Transferrin* (mg/dL)	Average Difference† (%)	Range (mg/dL)
Normal individuals	14	291 ± 8.5	−2.0 ± 1.3	220–340
Normal women with saturation <30%	9	283 ± 16.0	17.0 ± 2.0‡	249–360
Bone marrow transplant	9	291 ± 8.5	14.0 ± 3.1‡	114–256
Chronic liver disease	14	260 ± 23.9	10.0 ± 2.9‡	164–341
Malabsorption	6	210 ± 25.0	0.0 ± 1.9	188–296
Renal disease	5	243 ± 33.1	3.0 ± 2.9	155–324

*Means ± SEM of serum transferrin concentration measured immunochemically.

†For each patient, the % monoferric transferrin obtained from gels was subtracted from the % monoferric transferrin obtained from serum iron and transferrin measurements. The means ± SEM of the differences are shown.

‡Significantly different from 0, $P < .01$ (Student's *t* test).

ity of the molecule. Selected examples are summarized in Table 2. If the *N* and *C* sites are equal and there is no cooperativity of binding, 50% of the transferrin would be monoferric, 25% would be diferric, and the relative saturability is defined as 100%. Differences in binding constants of tenfold or greater would yield abnormal distributions readily detected by the gels, but would not significantly alter the saturability of the transferrin. Differences of 50-fold or greater would decrease the saturability.

Interestingly, our attempts to saturate, in vitro, the transferrin of one of the bone marrow transplant patients who had increased monoferric and decreased diferric transferrin resulted in only 76% iron saturation, using conditions that yielded 97% saturation of a normal serum. These results are consistent with a 50- to 100-fold difference between the *N* and *C* sites of transferrin (Table 2). The serum may contain an abnormal transferrin molecule, a possibility suggested in at least one previously reported case,[24] or it may harbor factors that alter iron loading. Further studies with purified transferrin from such patients will be necessary to distinguish between these possibilities.

Table 2. Effect of Changes in Binding Constants for Iron of *N*- and *C*-Terminal Binding Sites on Amounts of Monoferric and Diferric Transferrin and Their Relative Saturation

K1C	K2C	K1N	K2N	% Monoferric Transferrin at 50% Saturation*	% Diferric Transferrin at 50% Saturation*	Saturability*†
1.00	1.00	1.00	1.00	50	25	100
1.00	1.00	0.50	0.50	51	24	99
1.00	1.00	0.33	0.33	54	24	98
1.00	1.00	0.25	0.25	55	22	97
1.00	1.00	0.20	0.20	57	22	97
1.00	1.00	0.10	0.10	63	18	95
1.00	1.00	0.05	0.05	70	15	91
1.00	1.00	0.02	0.02	77	11	83
1.00	1.00	0.01	0.01	82	8	74

K1N, $[Tf\text{-}Fe_N]/[Tf][Fe]$; K2N, $[Tf\text{-}Fe_2]/[Tf\text{-}Fe_C][Fe]$; K1C, $[Tf\text{-}Fe_C]/[Tf][Fe]$; K2C, $[Tf\text{-}Fe_2]/[Tf\text{-}Fe_N][Fe]$, where Tf = apotransferrin; $Tf\text{-}Fe_N$ = *N*-terminal monoferric Tf; $Tf\text{-}Fe_C$ = *C*-terminal monoferric Tf.

*The percentages of monoferric transferrin ($X_N + X_C$) and diferric transferrin (X_D), and the percentage of saturation $[X_D + (X_N + X_C)/2]$ were calculated using the following equations from Chasteen and Williams (1981)[21]: $X_N = (1 + (K1C/K1N) + (1/K1N[Fe]) + (K2C[Fe]))^{-1}$; $X_C = (1 + (K1N/K1C) + (1/K1C[Fe] + K2N[Fe]))^{-1}$; $X_D = (1 + (K2C + K2N/K2CK2N[Fe]) + (K2CK1N[Fe]^2)^{-1})^{-1}$.

†Relative saturability = % saturation calculated using the relative amount of iron, which yields 100% saturation when all of the relative affinity constants are equal to 1.00 (line 1).

We have not determined the molecular basis for the elevation in monoferric transferrin in patients with cirrhosis of the liver, bone marrow transplant patients, or patients with iron deficiency anemia, but suspect that this observation provides further evidence for altered iron or transferrin metabolism in these patients.[25–29]

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