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Hematology

Bleomycin-detectable Iron Assay for Non-Transferrin-bound Iron in Hematologic Malignancies

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Background: **A microwell modification of the bleomycin assay for determining non-transferrin-bound iron (NTBI) was evaluated and compared with a chelation method.**

Methods: **The bleomycin assay reagent and sample volumes were halved, and measurements were done in microwell plates. Samples from patients treated for hematologic malignancies were studied. The chelation method was based on mobilization of NTBI with a chelator and measurement of the ultrafiltered iron-chelator complex. NTBI results were also compared with transferrin saturation and the distribution of transferrin iron forms by urea-polyacrylamide gel electrophoresis.**

Results: **The bleomycin assay intraassay imprecision (CV) was 7.7% and 8.2% and the interassay imprecision was 18% and 9.8% for a low (0.2 μmol/L) and a high (1.5 μmol/L) control, respectively. Hemolysis increased measured NTBI. A detection limit of 0.1 μmol/L was established based on the interference of nonvisible hemolysis and on accuracy studies. In patient samples, NTBI exceeded the detection limits only when transferrin saturation was >80%. Compared with the chelation method, the bleomycin assay gave clearly lower NTBI concentrations. The chelation method also gave positive results at <80% transferrin saturation. The recovery of iron added as ferric nitrilotriacetate to serum was 33% by the bleomycin assay and 64% by the chelation assay.**

Conclusions: **The microwell version of the bleomycin assay is reproducible. When hemolyzed samples were excluded, bleomycin-detectable iron was found only when the transferrin saturation was >80%, suggesting high specificity. Bleomycin-detectable iron constitutes only a portion of the NTBI measured by the chelation method.**

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Iron is bound with high affinity to transferrin, the iron carrier protein in serum. In some clinical conditions, transferrin becomes fully saturated, and non-transferrin-bound iron (NTBI)[4] appears in serum. NTBI is harmful because of its high redox activity and its potential for forming free radicals, thereby inducing cellular toxicity *(1, 2)*. It is also known that iron has a critical role in the growth of bacteria and fungi, and NTBI may promote the growth of microorganisms and predispose patients to septic infections. NTBI has generally been detected in disorders with iron-overload conditions, such as thalassemia *(3)* and hemochromatosis *(4, 5)*, and in certain other clinical conditions *(6–8)*. One group of patients who typically have NTBI includes hematologic patients undergoing high-dose chemotherapy *(9)*, particularly those treated with myeloablative therapy and stem cell transplantation *(10, 11)*.

Beginning with the chelation method described by Hershko et al. *(3)*, several assays have been developed to measure NTBI without interference of iron bound in a redox-inactive form to transferrin or to therapeutic chelating agents such as desferrioxamine. Two types of assays have been described: those in which loosely bound

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[4] Nonstandard abbreviations: NTBI, non-transferrin-bound iron; NTA, nitrilotriacetic acid; AAS, atomic absorption spectrophotometry; FeNTA, ferric nitrilotriacetic acid; PBS, phosphate-buffered saline; and PAGE, polyacrylamide gel electrophoresis.

iron is first mobilized with a chelating agent and the ultrafiltered complex is measured *(3, 12–14)*; and assays in which a biologic reagent forms a complex with redox-active iron, causing a biochemical reaction that can be measured *(15, 16)*. The most common chelating agent used in the chelation assays is nitrilotriacetate (NTA). The ultrafiltered chelated iron can be quantified by different methods, including HPLC, colorimetric measurement, and atomic absorption spectrophotometry (AAS). In the method first described by Breuer et al. *(13)*, oxalate is used for chelation of NTBI, and quantification is based on fluorescence measurement in microwells coated with desferrioxamine in the presence of a fluorescent metallosensor, calcein. The bleomycin assay developed by Gutteridge et al. *(15)* is based on the direct complexation of redox-active iron with the antibiotic bleomycin. The iron–bleomycin complex causes degradation of DNA, and the degradation products can be measured. The detected iron is generally named bleomycin-detectable iron. The bleomycin assay has been compared with another biochemical assay that uses the iron-requiring enzyme aconitase *(16)*, which gave results similar to the bleomycin method. Both the chelation methods and the bleomycin assay have been used for measuring NTBI in various clinical conditions.

One drawback of most chelation methods is the large amount (~1 mL) of serum needed for ultrafiltration, whereas the bleomycin assay requires only 100 μL. In this work, we further reduced the scale of the bleomycin assay and introduced a spectrophotometric measurement in microwell plates. Because very limited information has been available on the performance of the bleomycin assay concerning assay precision, detection limit, and interference, we evaluated the assay. We also compared the bleomycin assay with a chelation assay for the determination of NTBI in the serum of patients with hematologic malignancies.

Materials and Methods

Patient samples

Serum samples (n = 399) were collected from patients with hematologic malignancies undergoing allogeneic stem cell transplantation and treated with cyclophosphamide (120 mg/kg) and fractionated total body irradiation (12 Gy). The patients were enrolled in clinical studies for chelation of NTBI by an investigational apotransferrin product. For the comparative analysis of NTBI measured by the chelation assay, additional samples (n = 21) were analyzed from patients undergoing cytotoxic treatment with or without autologous stem cell transplantation. The samples were collected at the Helsinki University Central Hospital, Finland, and the studies were approved by the ethics committee of the hospital.

Microwell modification of bleomycin assay

The bleomycin assay was carried out according to the method described by Evans and Halliwell *(17)* with the following modifications. All reactions were carried out in one-half the volume, and the absorbance was measured at 535 nm rather than 532 nm, using a microwell plate reader. The reagent solutions, except the bleomycin, were treated overnight with Chelex (Bio-Rad), 300 mg in 10 mL or 800 mg in 20 mL of ascorbic acid solution, to remove any excess iron in the chemicals. To avoid iron contamination, only disposable plastic tubes were used. The assay components were added in the following order: 250 μL of 1 g/L DNA (type I DNA from calf thymus; Sigma); 25 μL of 1.5 kIU/L bleomycin sulfate (Sigma); 50 μL of 50 mmol/L $MgCl_2$; 25 μL of sample, calibrator, or blank; and finally, 50 μL of 8 mmol/L ascorbic acid (Merck). A predetermined amount of 25 mmol/L HCl (~7 μL) was added to adjust the pH of reagent mixture to 7.4. The mixture was incubated at 37 °C for 1 h to allow the iron–bleomycin complexes to bind to DNA, releasing malondialdehyde from DNA degradation. The reaction was stopped by the addition of 50 μL of 0.1 mol/L EDTA, and 250 μL of 10 g/L thiobarbituric acid (Sigma) and 250 μL of 250 mL/L HCl were added. The mixture was then incubated for 20 min at 80 °C to allow chromogen formation by the reaction of malondialdehyde with thiobarbituric acid. The samples were cooled to room temperature, 1.5 mL of butanol was added, and the chromogen was extracted into the organic phase by mixing. The samples were centrifuged for 20 min at 2500*g* to separate the phases, and 350 μL of the clear top phase was pipetted into microwell plates (Nunc Maxisorp). The absorbance was measured at 535 nm in a microplate reader (Titertek Multiscan RC; Labsystems). The samples were measured in parallel with a corresponding blank without added bleomycin. The absorbance of the blank was subtracted from that of each sample. The absorbance of the reagent blank was subtracted from the absorbances of the calibrators, and a calibration curve with 0.1–3 μmol/L iron (iron atomic absorption standard solution in 10 mL/L HCl; Sigma Aldrich) was calculated for each series. Data were transformed to logarithmic values before calculation of the calibration curve by linear regression to give weight to the calibrators with a low iron concentration (0.1–1 μmol/L) before curve fitting.

We prepared positive controls by adding ferric nitrilotriacetic acid (FeNTA) to pooled, complement-inactivated, sterile-filtered normal serum. A 1 mmol/L FeNTA solution was prepared from $FeCl_3$ and NTA (Sigma), in a 1:1 molar ratio. We added 30–40 μmol/L FeNTA to obtain serum with fully saturated transferrin and NTBI. The normal serum without added FeNTA was used as a negative control.

NTBI assay with iron chelator NTA

The NTBI assay with iron chelation and colorimetric quantification was carried out according to the method of Gosriwatana et al. *(14)*. The serum samples were prepared by adding 100 μL of 800 mmol/L NTA (pH 7.0), prepared from disodium and trisodium salts of NTA, to 900 μL of serum and allowed to stand for 30 min at room

temperature. Serum filtrates were collected by ultrafiltration in centrifugal filter devices with M_r 30 000 cutoff filters (Ultrafree-4; Millipore) that had been prerinsed with 10 mmol/L NTA and flushed with ultrapure water. For the colorimetric determination of iron, 400 μL of serum ultrafiltrate was diluted with 400 μL of 5 mmol/L MOPS buffer (pH 7.4), and 100 μL of 120 mmol/L thioglycolic acid (Sigma) was added, followed by 100 μL of 60 mmol/L bathophenanthroline disulfonic acid, disodium salt (Sigma). The mixture was then allowed to stand for 30 min at room temperature. The absorbance of 350-μL samples in microwell plates was measured at 535 nm, using a microplate reader. Calibrators with 0–20 μmol/L of the same iron calibrator as in the bleomycin assay were prepared in 80 mmol/L NTA, and a calibration curve was calculated by linear regression.

Chelated iron in the ultrafiltrates was also measured by graphite furnace AAS (Perkin-Elmer AAnalyst 600 equipped with Zeeman background correction). The samples and calibrators were diluted with ultrapure water, and $Mg(NO_3)_2$ was used as a matrix modifier. The accuracy and precision of the method was monitored with standard reference material Seronorm™ Trace Elements (Sero AS).

OTHER METHODS

Serum iron was measured by colorimetric measurement without deproteinization using ferene-S as the chromogen *(18)*. The interassay imprecision (CV) was 2.8% and 3.0% for a low (19.7 μmol/L) and a high (51.4 μmol/L) control, respectively. Serum transferrin was measured by an immunoturbidimetric method (Orion Diagnostica) with IMPRO/Nordic 96 calibrator (Labquality), which is standardized to the IFCC calibrator BRC-470. The imprecision of the controls was 3.2% for 0.97 g/L, 2.8% for 2.11 g/L, and 3.7% for 2.67 g/L. The percentage of transferrin saturation was calculated from the serum iron and transferrin concentrations, using the formula: serum iron (μmol/L)/transferrin (g/L) × 3.98. The formula is based on the maximal binding of 2 mol Fe^{3+}/mol of transferrin and a molecular weight of 79 570 for transferrin *(19)*. Hemoglobin in serum was measured by a colorimetric method with *o*-toluidine *(20)*. Serum ferritin was measured by a direct immunochemiluminometric method on an ACS:Centaur apparatus (Chiron Diagnostics).

The iron forms of transferrin were separated by urea polyacrylamide gel (6% acrylamide gels with 6 mol/L urea) electrophoresis according to Williams et al. *(21)* and visualized by immunoblotting. Serum samples with ~0.15 μg of transferrin were separated in 10 × 10-cm gels. Proteins were electroblotted from the gel onto polyvinylidene fluoride membrane (Immobilon-P; Millipore) in a transfer buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 200 mL/L methanol. The membrane was treated with 50 mL/L Tween in phosphate-buffered saline (PBS) overnight. Transferrin bands were visualized by immunostaining using rabbit anti-human transferrin IgG (Dako A/S) as the primary antibody in PBS containing 10 g/L bovine serum albumin and 0.5 mL/L Tween 20 for 2 h at room temperature. The blots were washed three times with PBS containing 0.5 mL/L Tween 20 and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immuno Research Laboratories) in PBS containing 10 g/L bovine serum albumin and 0.5 mL/L Tween 20 for 1 h at room temperature. After three additional washes with 0.5 mL/L Tween 20 in PBS, the blots were stained with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium color development solution (Immuno-Blot Alkaline Phosphatase Assay reagent set; Bio-Rad). The reaction was stopped with 100 mmol/L sodium acetate (pH 5) containing 5 mmol/L sodium EDTA for 5 min. The blots were washed with distilled water and dried.

Results

PRECISION AND ACCURACY OF THE BLEOMYCIN ASSAY

We prepared a calibration curve, using pure 0.1–10 μmol/L iron calibrator in water; the calibration was linear up to 6 μmol/L. In the standardized assay, we used iron calibrators in the range of 0.1–3 μmol/L (Fig. 1) and determined the calibration curve by linear regression of logarithmic values. The sensitivity of the assay for the iron calibrator was 0.316 absorbance units per μmol/L. The assay precision was measured using a high and a low serum control, which were prepared by adding FeNTA to normal serum to saturate transferrin and form different concentrations of NTBI (Table 1). The assay detection limit was determined by use of a normal serum control without added iron (n = 10). The limit of detection, calculated as 3 SD above the mean of the negative control, was 0.05 μmol/L.

To determine the accuracy of the bleomycin assay, iron in the form of FeNTA was added to normal serum in increments of 1 μmol/L. The transition to full transferrin saturation was confirmed by urea-polyacrylamide gel

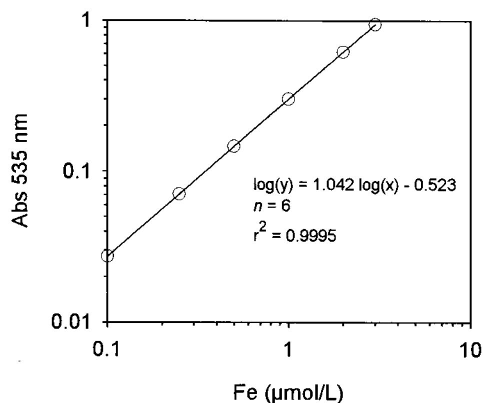

Fig. 1. Calibration curve for the bleomycin assay.

Pure iron calibrator in water was measured by the assay, and the iron concentration was plotted against the measured absorbances. The calibration curve was calculated by linear regression of the logarithmic values.

Table 1. Bleomycin assay precision.[a]

	n	CV, %	Mean, µmol/L	95% confidence interval
Intraassay				
Low control	10	7.7	0.21	0.20–0.22
High control	10	8.2	1.47	1.40–1.54
Interassay				
Low control	28	18	0.21	0.19–0.22
High control	28	9.8	1.54	1.48–1.59

[a] Controls were prepared by adding FeNTA to normal serum in amounts sufficient to saturate transferrin and produce different concentrations of NTBI.

electrophoresis (PAGE). When the transferrin was found only in the iron-saturated form, the NTBI was ≥0.1 µmol/L, whereas the concentration was <0.1 µmol/L when nonsaturated forms of transferrin could be detected by urea-PAGE. These results were from three experiments using different starting serum pools, with slight variations in the points for full transferrin saturation attributable to variations in iron and transferrin concentrations. A typical curve is presented in Fig. 2. The results suggested that the limit of detection of the assay was 0.1 µmol/L. The NTBI concentration increased more steeply after the point of full saturation of transferrin. After the transferrin saturation, the slope of the curve indicated that ~33% of the added iron was measured as NTBI.

INTERFERENCE STUDIES

To investigate the possible interference of hemolysis in the bleomycin assay, we mechanically caused different degrees of hemolysis in blood samples before separation of the serum and determined the percentage of transferrin saturation and concentrations of hemoglobin, NTBI, and iron forms by urea-PAGE (Fig. 3A). Hemolysis did not increase the amount of total iron, and the transferrin saturation remained the same. Serum hemoglobin in the samples ranged from 60 mg/L in samples without visible

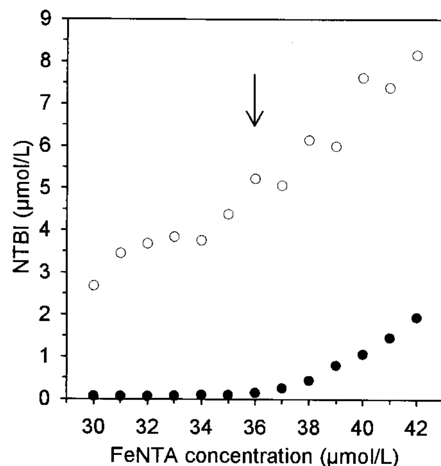

Fig. 2. Recovery of NTBI in normal serum with iron added as FeNTA.

NTBI was measured by the bleomycin assay (●) or the chelation method (○). The *arrow* indicates the point of full transferrin saturation determined by urea-PAGE.

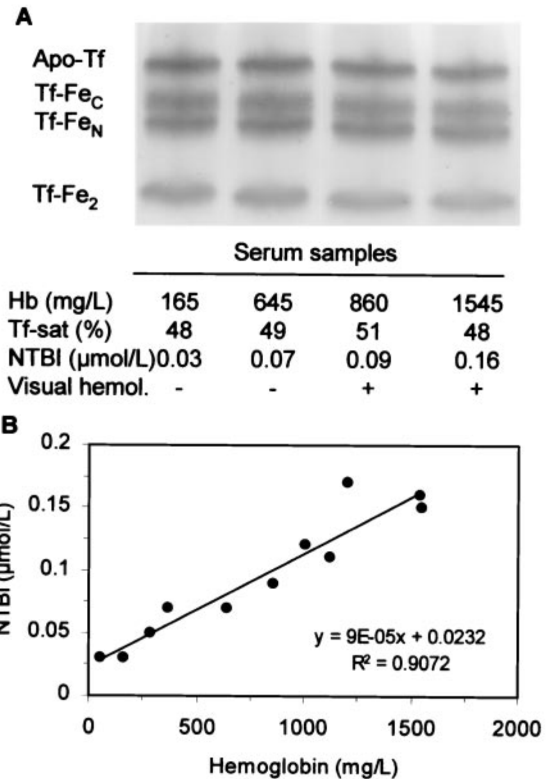

Fig. 3. Influence of hemolysis on NTBI measured by the bleomycin assay.

(*A*), influence of hemolysis on transferrin iron forms, as analyzed by urea-PAGE, and on hemoglobin (*Hb*), calculated transferrin saturation (*Tf-sat*), and NTBI in serum. *Apo-Tf*, apotransferrin; *Tf-Fe$_C$*, monoferric transferrin with iron in C-lobe; *Tf-Fe$_N$*, monoferric transferrin with iron in N-lobe; *Tf-Fe$_2$*, diferric transferrin; *hemol*, hemolysis. (*B*), dependence of NTBI on the hemoglobin concentration in serum. Serum samples were prepared from blood hemolyzed to different extents ex vivo.

hemolysis to 1550 mg/L in samples with clearly visible hemolysis. A clear correlation between hemoglobin and the NTBI concentration could be seen (Fig. 3B). NTBI concentrations up to 0.07 µmol/L could be measured when the hemolysis was not visually detectable and the hemoglobin was 290–645 mg/L. Any possible nonspecific effects attributable to hemolysis were corrected by use of the sample blank measured without added bleomycin, as described in *Materials and Methods*. It was concluded that, to exclude interference caused by nonvisible hemolysis, a limit of detection close to 0.1 µmol/L should be used.

We found no interference of serum ferritin in the samples. Patient serum samples for ferritin analysis were taken 4 days before the samples for the bleomycin assay. At ferritin concentrations of 5–2470 µg/L (mean ± SD, 568 ± 733 µg/L; n = 20), all corresponding NTBI results were <0.1 µmol/L. The reference intervals for ferritin were 7–100 µg/L for women and 15–300 µg/L for men. Even the high ferritin concentrations (>500 µg/L) found in one-third of the studied samples did not give detectable NTBI.

The interference of the cyclophosphamide used for

treatment of the patients was studied in normal serum and in patient sera with detectable NTBI. Cyclophosphamide was added in concentrations of 0.1–1.5 g/L, where the high concentration corresponded to a maximal theoretical concentration of the drug in serum. Cyclophosphamide did not interfere with NTBI results: in normal serum, NTBI was not detectable, and in the positive patient sera, the NTBI concentrations remained the same.

DETERMINATION OF NTBI IN PATIENT SERUM SAMPLES

NTBI concentrations were measured in 399 patient samples by the bleomycin assay, and the results were compared with transferrin saturation values (Fig. 4A). The NTBI varied between 0 and 0.97 μmol/L in the samples without visible hemolysis, whereas the highest value measured, 1.67 μmol/L, was in a hemolyzed sample. On the basis of our results for assay accuracy and interference of hemolysis, we used a limit of detection of 0.1 μmol/L for the patient samples. NTBI above the detection limit was found only in samples with transferrin saturation >80%. At transferrin saturation <80%, all samples had NTBI below the detection limit (Table 2). For comparison, we also calculated the number of samples that had <80% transferrin saturation and NTBI concentrations above the detection limit (0.05 μmol/L) determined from the negative control; we found 15 such samples (4% of total). The samples with <0.1 μmol/L NTBI and transferrin saturation >80% were studied by urea-PAGE, and most (88%) had monoferric transferrin in addition to the diferric form, indicating that transferrin was not completely saturated. Of the positive samples with visual hemolysis, one sample with 0.12 μmol/L NTBI and 80% transferrin saturation contained iron-free apotransferrin and was therefore considered a false positive. The results indicate a high apparent specificity of the assay, with no false positives at transferrin saturation <80% when hemolyzed samples were excluded.

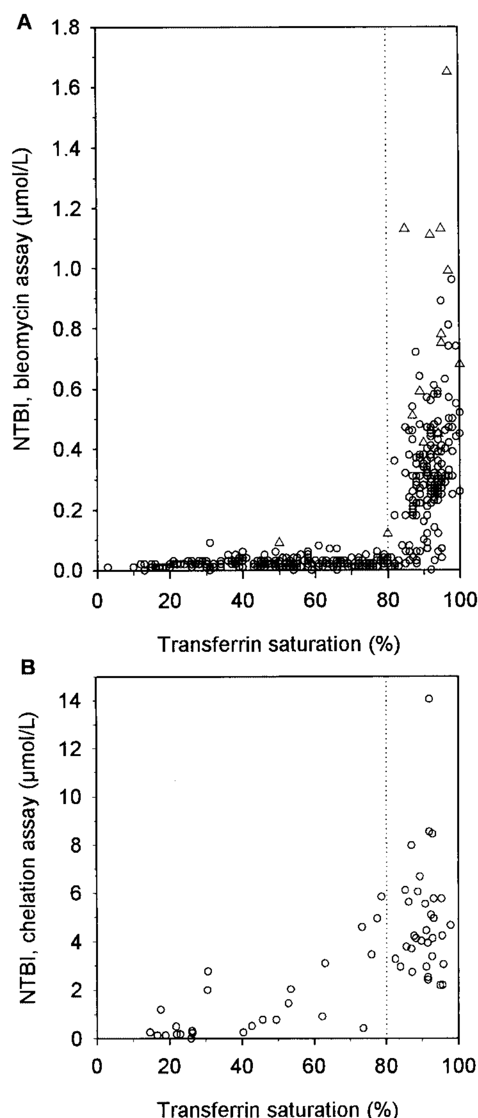

Fig. 4. Correlation between transferrin saturation and NTBI in serum from patients with hematologic malignancies.

NTBI was measured by the bleomycin assay (*A*) or by the chelation method (*B*). ○, samples without visible hemolysis; △, samples with visible hemolysis.

COMPARISON WITH THE CHELATION METHOD

NTBI concentrations in patient samples were studied with another method that was based on the chelation of the NTBI in serum with NTA and colorimetric quantification of the chelated iron in the ultrafiltrate. The sensitivity of the colorimetric assay was 0.01 absorbance units per μmol/L and was clearly lower than that of the bleomycin assay. The limit of detection, calculated as 3 SD above the mean of a normal control serum (n = 10), was 1.5 μmol/L. The limit of detection was clearly higher than the corresponding value for the bleomycin assay (0.05 μmol/L) and reflected the larger variation of the results obtained by the chelation assay for the normal serum control (0.08 ± 0.48 μmol/L, mean ± SD).

We assayed the recovery of iron in serum samples in the same manner as in the bleomycin assay, using serum samples with added FeNTA (Fig. 2). The mean recovery for added FeNTA in three separate determinations was 64%, which was approximately twofold higher than the recovery of the bleomycin assay. However, the chelation method already showed gradually increasing positive NTBI values for the samples with added FeNTA before

Table 2. NTBI by the bleomycin assay compared with transferrin saturation in patients with hematologic malignancies.

Bleomycin assay	n	%
Total number of samples studied	399	100
NTBI positive (≥0.1 μmol/L)	156	39
NTBI negative (<0.1 μmol/L)	243	61
Positive samples with transferrin saturation <80%	0	0
Negative samples with transferrin saturation >80%	31	8

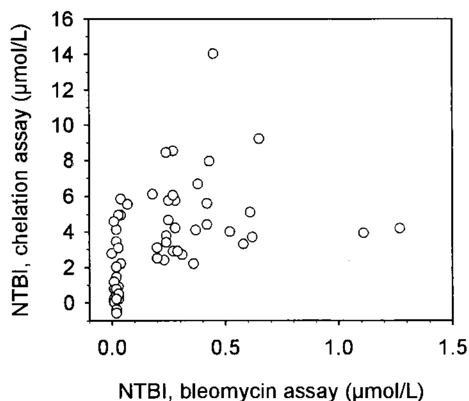

Fig. 5. Comparison of serum NTBI results obtained by the bleomycin assay and the chelation method in patients with hematologic malignancies.

the full transferrin saturation point, as determined by urea-PAGE (Fig. 2).

We studied 61 patient samples in parallel, using the bleomycin assay and the chelation-based NTBI assay, and found considerable variation between the two assays (Fig. 5). All samples that were positive in the bleomycin assay also were positive in the chelation assay, whereas one-third (11 of 30) of the samples with no bleomycin-detectable NTBI were positive in the chelation assay. A majority (8 of 11) of these samples had transferrin saturation <80% (Fig. 4B), and nonsaturated forms of transferrin could be detected in urea-PAGE. In samples with NTBI concentrations above the detection limit, no clear association between the concentrations of the two methods could be found, but clearly, higher absolute concentrations, up to 14 μmol/L, could be measured by the chelation method (Fig. 5). Because the colorimetric quantification method had a low sensitivity, we measured the amount of chelated NTBI in parallel, using AAS to verify the results of the colorimetric method. The limit of detection of the AAS method, calculated as 3 SD above the mean of the standard without iron, was 0.02 μmol/L, and the interassay imprecision (CV) was 2.2%. The results indicated that similar values for NTBI were obtained by both quantification methods [$NTBI_{AAS} = 0.9373 (NTBI_{colorimetric}) + 0.7057$; $r^2 = 0.856$; $S_{y|x} = 0.693$; n = 21].

Discussion

Our assay evaluation demonstrates that the microwell modification of the bleomycin assay can be carried out reproducibly using one-half the amount of reagents and serum compared with the original assay *(17)* and makes it possible to study large numbers of patient samples. In interference studies, we found that hemolysis increased the NTBI measured by the bleomycin assay; we also found a clear correlation between serum hemoglobin and NTBI. Slightly hemolyzed sera with no clearly visible hemolysis but with increased hemoglobin concentrations had measured NTBI concentrations of 0.05–0.1 μmol/L. In recovery studies, full transferrin saturation occurred when the measured NTBI was 0.07–0.1 μmol/L. On the basis of these results, we used a detection limit of 0.1 μmol/L as the positive cutoff for bleomycin-detectable iron in serum.

An earlier report *(15)* indicated that pure hemoglobin (1000 mg/L) does not interfere with the bleomycin assay. In our study, interference was observed in serum samples subjected to mechanical hemolysis during sample preparation; the interference may have been caused by heme released from hemoglobin or its degradation products. In line with our results, Pepper et al. *(22)* reported that some cardiopulmonary bypass patients who were subject to hemolysis during extracorporeal circulation had redox-active iron in their serum as measured by the bleomycin assay. Because the hemolysis took place in vivo in circulation, the presence of redox-active iron species in the serum of these patients probably was clinically important. Therefore, the cause of hemolysis should always be considered when evaluating the importance of redox-active iron in serum samples.

When we analyzed 399 serum samples from patients with hematologic malignancies by the bleomycin assay, we found NTBI-positive samples only when transferrin saturation was >80%, which suggested high specificity. The measured values were between 0 and 1 μmol/L. Carmine et al. *(23)* found similar NTBI concentrations in patient samples, as measured by the bleomycin assay, whereas others have reported measured concentrations of 20–28 μmol/L *(9, 24)*. The higher total concentrations were found with an earlier version of the bleomycin assay *(15)* without the final butanol extraction step, which apparently reduces the risk of interference from precipitated proteins in the spectrophotometric measurement. Because these patients often have high serum ferritin concentrations, the ferritin interference was studied. No interference was found from ferritin, which was in accordance with results obtained using another type of NTBI assay based on iron chelation *(10)*. The cyclophosphamide used for myeloablative treatment did not interfere with the bleomycin assay after in vitro addition. Furthermore, a sample blank value determined without the addition of bleomycin is always subtracted from each sample to assess possible interference from chemotherapy agents.

Transferrin saturation was calculated from the transferrin and iron determinations, which had assay CVs of 2.8–3.7%. An estimate of 4.8% for the imprecision of the transferrin saturation determination could be calculated from the respective assay CVs *(25)*, indicating an acceptable precision for this method. The use of an immunochemical assay to measure transferrin in combination with a colorimetric iron assay is considered a reliable method to calculate transferrin saturation, provided that the correct molecular weight of transferrin is used *(26)*. It has been suggested that this method shows less variability than the total iron-binding capacity method, especially at high ferritin concentrations *(26, 27)*. In this study, the

point of full transferrin saturation was confirmed by urea-PAGE, in which only the diferric form of transferrin is detected at full saturation.

The bleomycin assay was compared with another method, which is based on the mobilization of NTBI with the iron chelator NTA and separation of the chelated iron by ultrafiltration. The iron in the ultrafiltrate was quantified by a chromogenic assay or AAS. The NTBI concentrations were in a range (0–10 μmol/L) similar to those found by others using the same type of assay *(10, 14)*. The concentrations of chelatable NTBI showed variations in comparison with the bleomycin-detectable NTBI concentrations, with higher NTBI concentrations measured by the chelation assay. However, we found that unlike the bleomycin assay, in which positive samples were detected only when transferrin saturation was >80%, the chelation method also detected NTBI in some serum samples when the transferrin saturation was <80%. The presence of iron-free and monoferric transferrin in urea-PAGE confirmed the partial transferrin saturation in these samples.

The true nature in which NTBI exists in serum has been a matter of discussion. Ferric iron is virtually nonexistent as a free ion at neutral pH and readily forms colloidal, mostly insoluble hydroxide forms. On the other hand, serum contains small molecule-sized chelates such as citrate, which is considered an important complexing agent of NTBI *(5)*. NTBI most likely also binds loosely to albumin *(28)* and to other plasma proteins, amino acids, peptides, and sugars. It has been suggested that the NTBI pool is heterogeneous with iron complexes with different affinities for chelators or iron scavengers at different proportions. The use of a chelator to mobilize NTBI in serum samples evidently is a reason for the higher NTBI concentrations measured by the chelation-based assay. The chelator probably mobilizes iron from complexes in which it is not available for the bleomycin reagent. Gosriwatana et al. *(14)* found that NTA was able to mobilize iron from albumin, but that it also mobilized small amounts of iron from other proteins, such as transferrin (~0.5 μmol/L) and ferritin (~0.3 μmol/L). The use of the chelating agent could also explain our finding of detectable NTBI in serum samples with <80% transferrin saturation if the chelating agent had mobilized a small amount of transferrin-bound iron. Further evidence of the risk of mobilizing a portion of transferrin-bound iron when using a chelating agent was provided by Breuer et al. *(13)*, who used oxalate to chelate NTBI and shuttle it to immobilized desferrioxamine for detection. They found that in samples with transferrin saturation >60%, a substantial part (1–5%) of transferrin-bound iron was measured in the presence of oxalate, but not in its absence.

The recovery of added iron was higher (64%) in the chelation method than in the bleomycin method (33%), but neither assay could measure all of the FeNTA iron added to serum. FeNTA has been widely used to saturate transferrin in in vitro studies because of the better solubility of ferric iron in complex with NTA and its rapid binding to transferrin *(29)*. Addition of FeNTA to normal serum produced fully saturated transferrin, which was confirmed by urea-PAGE. However, it is not clear in which form the excess iron added to serum occurred and whether part of it formed insoluble ferric hydroxides. Such insoluble iron complexes may not be mobilized, even by NTA.

It is reasonable to conclude that the occurrence of NTBI is unlikely in serum with partial transferrin saturation, provided that there is no specific reason that iron binding by transferrin would be impaired, such as the severe ferrooxidase deficiency typically found in preterm babies *(30, 31)*. The presence of NTBI at partial transferrin saturation has, however, been reported for patients with hemochromatosis measured both with the bleomycin assay and the chelation method *(14, 32)*. It is therefore possible that some patient groups can have NTBI, or a portion of it, in a form that is not readily bound to transferrin. Our results with the bleomycin assay for patients with hematologic malignancies undergoing allogeneic stem cell transplantation did not indicate that NTBI might be present in any situations other than at >80% transferrin saturation unless the samples were clearly hemolyzed. The NTBI measured by the bleomycin assay is capable of binding to bleomycin and generating free radicals; it therefore is considered a measure of the redox activity of NTBI. The bleomycin assay thus specifically detects redox-active NTBI in serum samples, although probably cannot measure the total amount of NTBI.

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