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Effective binding of free iron by a single intravenous dose of human apotransferrin in haematological stem cell transplant patients

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Summary. Myeloablative treatment results in iron accumulation and the appearance of non-transferrin-bound iron (NTBI) in the circulation, which may contribute to treatment-related organ damage and susceptibility to infections. The aim of this study was to investigate the efficacy of human apotransferrin in the binding of NTBI in patients receiving an allogeneic stem cell transplant after myeloablative conditioning. A single intravenous 100 mg/kg dose of apotransferrin was given to six adult patients on d 3 after the transplantation. Initially, all patients had serum transferrin saturation above 80% and NTBI in their serum. After the apotransferrin injection, serum NTBI became undetectable in all patients and transferrin saturation decreased to 30–50%. Serum transferrin increased by an average of 1·95 g/l. The administered apotransferrin was subsequently converted into monoferric and diferric transferrin forms. NTBI reappeared and transferrin saturation again exceeded 80% 12–48 h after the injection in four patients and after 6 d in one patient. NTBI remained non-detectable for the whole 12 d follow-up period in one patient. The apotransferrin injection was well tolerated and no adverse events with probable association with the apotransferrin were observed. Repeated apotransferrin infusions might completely eliminate NTBI and iron-induced toxicity during myeloablative therapy.

Keywords: apotransferrin, NTBI, iron, stem cell transplantation, transferrin saturation.

Transferrin is the major iron-carrier protein in human plasma and extracellular space in tissues. It binds two ferric ions with high affinity and prevents the participation of the bound iron in redox reactions and generation of hydroxyl radicals (Baldwin *et al*, 1984). Further, transferrin-bound iron cannot be utilized by most bacteria and fungi, and transferrin is therefore an important antimicrobial factor in plasma (Bullen, 1981; Weinberg, 1984). In a healthy individual, transferrin iron saturation is 20–35% and redox-active, non-transferrin-bound iron (NTBI) cannot be detected in the serum. Several studies have indicated that high-dose chemotherapy of haematological malignancies is associated with an increase in the serum total iron content, which often exceeds the iron binding capacity of transferrin and results in the appearance of NTBI in the serum of the patients (Halliwell *et al*, 1988; Gordeuk & Brittenham, 1992; Harrison *et al*, 1994; Carmine *et al*, 1995). Complete transferrin saturation and the simultaneous presence of NTBI are particularly common during myeloablative therapy and stem cell transplantation (SCT) (Bradley *et al*, 1997; Dürken *et al*, 1997; Sahlstedt *et al*, 2001).

NTBI has been associated with two kinds of adverse events. First, several lines of evidence suggest that NTBI is cytotoxic (Anderson, 1999), and it has been suggested to play a part in the pathogenesis of mucosal and liver injuries associated with high-dose chemotherapy (Carmine *et al*, 1995; Beare & Steward, 1996). Second, NTBI may predispose the neutropenic and immunosuppressed patient to septic infections by opportunistic bacteria and fungi, which are dependent on free iron for growth (Harrison *et al*, 1994; Iglesias-Osma *et al*, 1995). Currently, there are no safe and effective means for the prevention of the appearance of NTBI in the patients undergoing myeloablative conditioning for SCT. Low-molecular-weight iron chelators like desferrioxamine display dose-related toxicity (Porter *et al*, 1996) and may increase the risk of bacterial and fungal infections (Weinberg, 1999). Theoretically, administration of iron-free apotransferrin to the patients could keep the accumulating iron in a transferrin-bound form

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until uptake by the erythroid marrow recovers. The aim of this study was to investigate the efficacy of a single intravenous dose of a human apotransferrin product in the binding of NTBI in SCT patients, and to perform initial pharmacokinetic and tolerance analyses.

MATERIALS AND METHODS

Patients. Seven consecutive patients with a haematological malignancy, who received conditioning with cyclophosphamide 120 mg/kg and fractionated total body irradiation of 12 Grays, and whose serum transferrin was < 2·00 g/l and transferrin saturation > 80% 3 d before the SCT, were enrolled (Table I). Patients with clinically significant cardiovascular or pulmonary disease or renal insufficiency were excluded. Patient 3 was excluded because of hypertensive blood pressure immediately prior to the planned administration of the study drug. The study was approved by the Ethics Committee of the Helsinki University Central Hospital and an informed written consent was obtained from all patients before enrolment.

Study drug. The study drug contained purified human plasma apotransferrin manufactured by the Finnish Red Cross Blood Transfusion Service (von Bonsdorff *et al*, 2001). The product was virus inactivated (solvent detergent treatment) and filtered with a 15 nm virus removal filter. Vials containing 1 g of the freeze-dried product were dissolved with 20 ml of water to obtain a 5% solution for injection.

Administration of study drug and follow-up. The primary endpoint of the study was the effect of a single apotransferrin injection on serum transferrin and iron parameters. A dose of 100 mg/kg of apotransferrin was administered as a slow injection, lasting about 30 min, to a deep vein catheter 3 d after SCT. The day of the study-drug administration was designated as d 0. Serum transferrin, total iron, NTBI, transferrin saturation and transferrin iron forms were determined from blood samples collected prior to the study drug administration and at 15 min, 2 h and 12 h after the study drug. Thereafter, the samples were collected daily for the first 6 d after the study drug administration and then on d+8, +10 and +12.

The immediate tolerance was monitored during the 2 h following the injection by blood pressure, pulse, temperature and respiratory rate recordings. The safety of the study drug was analysed by monitoring blood counts, liver and renal function, as well as electrolytes in the routine samples until d +10.

Laboratory methods. Serum total iron was measured using a colorimetric ferene-S method (reference range 8–30 μmol/l for women and 10–31 μmol/l for men). Serum transferrin was determined by an immunoturbidimetric method (reference range 1·75–3·13 g/l). The transferrin saturation was calculated by the following formula: serum iron (μmol/l)/serum transferrin (g/l) × 3·98. Serum ferritin was measured using the Ciba Corning ACS Ferritin chemiluminometric immunoassay with a reference range of 15–230 μg/l for men and 10–150 μg/l for women. Serum NTBI was determined with the bleomycin assay (Evans & Halliwell, 1994). The samples were measured in parallel with a corresponding blank without the addition of bleomycin. The absorbance value of the blank was subtracted from each sample absorbance value. The reagent blank value was subtracted from the absorbance values of the standards, and a standard curve between 0·1 and 3 μmol/l was calculated by linear regression from each series. According to a validation study, the limit of detection for NTBI was 0·1 μmol/l (von Bonsdorff *et al*, 2002). The iron forms of transferrin were analysed by urea polyacrylamide gel electrophoresis (6% acrylamide gels with 6 mol/l urea) according to Williams & Moreton (1980). Serum samples were precipitated with rivanol and samples corresponding to 2 μl of serum were run in 10 × 10 cm gels. Proteins were visualized with Coomassie brilliant blue staining and the proportions of the different transferrin iron forms were determined by scanning with a laser densitometer.

RESULTS

The serum transferrin level before the apotransferrin injection was below the lower reference limit in all patients, the mean being 1·51 g/l (range 1·07–1·74 g/l). After the intravenous injection of 100 mg/kg (99–106 mg/kg) of

Table I. Patient characteristics.

Patient	Age (years)	Sex	Weight (kg)	Donor	Haematological diagnosis
1	48	M	76	Sibling	Acute lymphoblastic leukaemia
2	29	M	81	Unrelated	Acute myeloblastic leukaemia
3*	19	F	53	Sibling	Acute myeloblastic leukaemia
4	56	F	70	Sibling	Myelodysplastic syndrome, refractory anaemia
5	44	F	71	Sibling	Chronic myelogenous leukaemia
6	34	M	75	Unrelated	Myelodysplastic syndrome, refractory anaemia with excess blasts in transformation
7	39	F	71	Sibling	Chronic myelogenous leukaemia

*The study drug was not given to patient 3 because of hypertensive blood pressure.

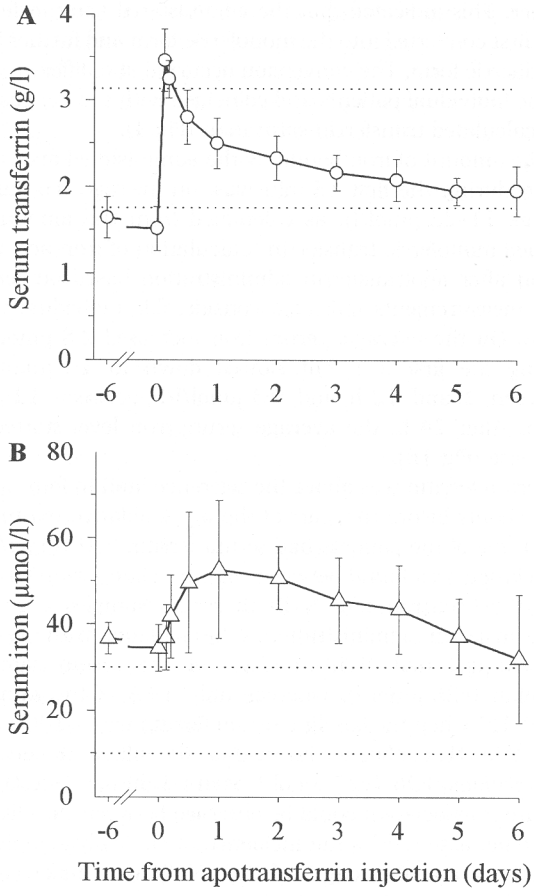

Fig 1. Serum transferrin (A) and total iron (B) in the six study patients before and after the intravenous injection of 100 mg/kg apotransferrin. The mean and SD are shown. The apotransferrin injection was administered on d 0. Dotted lines show the reference ranges.

apotransferrin, the serum transferrin concentration increased by an average of 1·95 g/l (1·48–2·42 g/l) peaking at the 15 min sample. The mean serum transferrin in the 15 min sample after the injection was 3·46 g/l (3·06–4·04 g/l), then it declined rapidly during the d 1, after which the decline occurred more slowly (Fig 1A). The provisional elimination-phase half-life calculated from a semi-logarithmic slope of the mean serum transferrin values between d 1 and 5 was approximately 4·8 d. The effect of endogenous transferrin turnover was not taken into consideration in the calculation.

The apotransferrin injection resulted in a temporary rise in the serum total iron level in all six patients (Fig 1B), although the extent and duration of the iron peak showed considerable variation. The mean serum iron level rose from 35 μmol/l (24–38 μmol/l) prior to the apotransferrin injection to a peak value of 53 μmol/l (26–66 μmol/l) approximately 24 h after the injection. The calculated transferrin saturation decreased from the mean level of 91% (87–94%) to 42% (30–49%) 15 min after the apotransferrin injection. After 2 h, the transferrin saturation started to increase again in all patients, but the course varied considerably in individual patients (Fig 2). The saturation level exceeded 80% in three patients 12–24 h after the apotransferrin injection, in one patient 2 d after the apotransferrin injection and in one patient 6 d after the apotransferrin injection. In patient 5, the transferrin saturation did not exceed 80% for the whole 12 d follow-up period.

The sera of all patients were positive for NTBI before the apotransferrin injection. Immediately after the injection, serum NTBI became undetectable in all patients. In five patients, NTBI later reappeared in the serum and was closely associated with the increase of the transferrin saturation above 80% (Fig 2). NTBI remained undetectable in patient 5 for the whole 12 d follow-up.

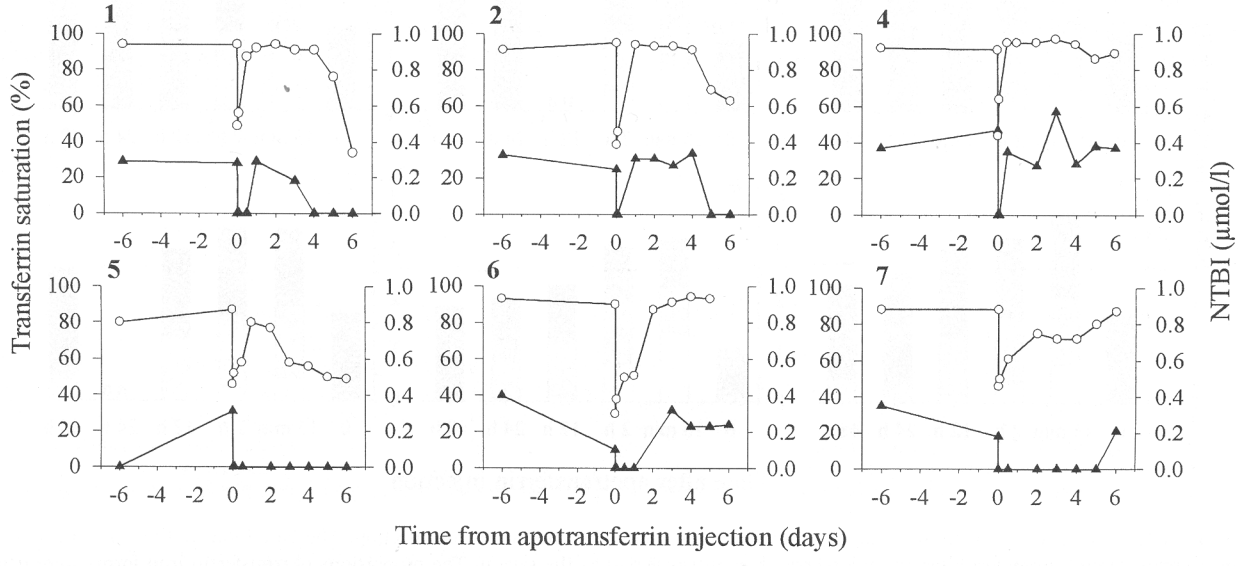

Fig 2. Serum transferrin saturation (○) and NTBI (▲) in the six study patients before and after the apotransferrin injection. NTBI was measured by the bleomycin assay.

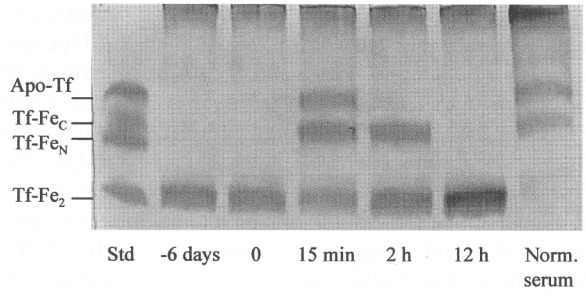

Fig 3. Transferrin iron forms of patient 4, analysed by urea gel electrophoresis before (d −6 and d 0) and after (15 min, 2 h, 12 h) the apotransferrin injection. First lane: transferrin standard containing iron-free (Apo-Tf), monoferric (Tf-Fe$_C$, Tf-Fe$_N$) and diferric forms (Tf-Fe$_2$); last lane: normal serum.

The conversion of the administered apotransferrin into monoferric and diferric transferrin forms was monitored by urea gel electrophoresis in sequential serum samples taken after the injection (Fig 3). At 15 min after the injection, iron-free and monoferric forms appeared in the sera of all patients. The amount of the diferric transferrin remained about the same in five patients but was reduced in one patient (patient 1). This suggested that most of the iron bound by the administered apotransferrin represented NTBI and was not shuttled from the fully saturated endogenous transferrin. At the later time points, the amount of the diferric form increased, while the iron-free form disappeared first and the monoferric form remained detectable somewhat longer. This indicated that the administered apotransferrin was first converted into the monoferric form and further into the diferric form. The conversion occurred at a different rate in the individual patients and coincided with the increase in the calculated transferrin saturation (Fig 4).

The amount of iron bound by the administered apotransferrin during the first 15 min was, on average, 16 μmol/l (range 11–22 μmol/l), as calculated from the amount of formed monoferric transferrin. Calculation of iron accumulation after apotransferrin administration based on serum iron measurements indicated considerable individual variation. On the average, serum iron increased 2·8 μmol/l/h during the first 2 h, but slowed down to 1·1 μmol/l/h between 2 and 12 h and 0·3 μmol/l/h between 12 and 24 h. After 24 h, the average serum iron level started to decrease (Fig 1B).

Serum ferritin was above the reference limit in four of the six patients before the start of the myeloablative treatment and three of the patients had serum ferritin > 1000 μg/l. A correlation was found between high baseline serum ferritin and the occurrence of NTBI in serum samples after the apotransferrin administration. The patients with ferritin > 1000 μg/l had NTBI in 72% of the serum samples (median 0·26 μmol/l), whereas only 19% of the samples had NTBI when the baseline serum ferritin was < 1000 μg/l ($P < 0·0001$, confidence interval for the difference between the medians 0·06–0·27 μmol/l, Mann–Whitney *U*-test). No apparent association could be observed between the clinical outcome, assessed by the incidence of clinically significant infections, and the length of time NTBI was undetectable after the apotransferrin injection.

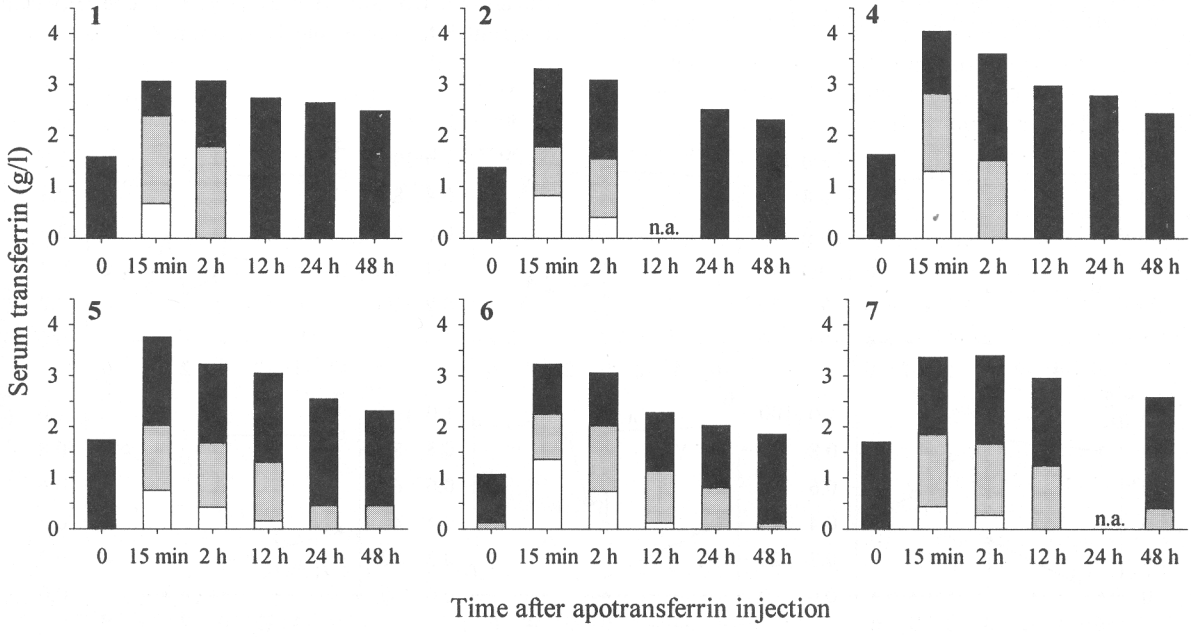

Fig 4. Transferrin iron forms in the six study patients before and after the apotransferrin injection. The bars indicate the concentrations of apotransferrin (white), monoferric (grey) and diferric (black) transferrin in the serum. The proportions of transferrin iron forms were determined by urea gel electrophoresis and their concentrations were calculated from the serum transferrin concentration. n.a., sample not available.

No serious adverse events occurred in the study patients during the 12 d follow-up period. No clinically significant changes in the vital signs occurred in association with the injection and the immediate general tolerability of the apotransferrin injection was good. No adverse events considered by the investigator to have possible association with the test drug occurred, with the exception of a mild, spontaneously subsiding chilly feeling in one patient, which was considered to have an improbable association with the study drug.

DISCUSSION

The present study demonstrated the feasibility of the binding of NTBI by an intravenous injection of apotransferrin in patients undergoing myeloablative conditioning for allogeneic SCT. The apotransferrin was given as a single dose of 100 mg/kg on d 3 after the transplantation to six adult patients. In a previous follow-up study of 10 patients undergoing allogeneic SCT, NTBI was detectable in the majority of patients for approximately 2 weeks, from 4 d before the transplantation up to 11 d post transplant (Sahlstedt *et al,* 2001). Accordingly, all the patients in the present study had a transferrin saturation of more than 80% and were positive for NTBI before the apotransferrin injection. As a result of the single apotransferrin injection, NTBI disappeared from the sera of all patients, indicating that it was effectively bound by the administered apotransferrin. Another indication of iron binding by the administered apotransferrin was the conversion of transferrin into monoferric and diferric forms in the sequential serum samples after the apotransferrin injection.

NTBI remained undetectable after the single apotransferrin injection for a variable time, ranging from a few hours up to several days. In most patients, NTBI reappeared 12–48 h after the apotransferrin injection. For complete prevention of NTBI during the whole SCT period, repeated administrations or continuous infusion of apotransferrin is evidently required. To our knowledge, no other iron-chelating agents have been studied in SCT patients, but desferrioxamine has been extensively studied in chronic iron overload diseases, such as hereditary haemochromatosis and thalassaemia major. It has been shown that some of the NTBI in haemachromatosis patients is not effectively chelated with desferrioxamine (Breuer *et al,* 2001) and the presence of NTBI in patient sera without full transferrin saturation has been reported (Aruoma *et al,* 1988; Gosriwatana *et al,* 1999; Loreal *et al,* 2000). NTBI probably comprises heterogeneous iron complexes which may differ in their binding to chelating agents and whose proportions may vary in different clinical conditions (Breuer *et al,* 2000). The bleomycin assay used in the present study measures redox-active iron and this assay has given positive results in SCT patients only when transferrin saturation exceeds 80% (von Bonsdorff *et al,* 2002). The disappearance of bleomycin-detectable NTBI after the apotransferrin injection indicated that the redox-active iron in the serum of SCT patients was in a form that was effectively bound by transferrin.

The bleomycin assay gives clearly lower values for NTBI than other methods based on mobilization of NTBI with a chelator, and determination of the ultrafiltered iron–chelator complex (von Bonsdorff *et al,* 2002). We used the bleomycin assay because it has a high specificity for NTBI and does not measure ferritin-bound iron. In the present study, we could evaluate the true level of NTBI from the amount of iron bound to the administered apotransferrin based on the shift of apotransferrin into the monoferric form. The average level (16 μmol/l) represented about 50% of total serum iron in the patients, which was slightly higher, but in the same order of magnitude, as that found by the chelation method in SCT patients (von Bonsdorff *et al,* 2002). Serum iron accumulation continued after apotransferrin administration at a variable rate and levelled off at 24 h in most patients. This may represent further iron mobilization by transferrin from insoluble complexes and release from catabolism of senescent erythrocytes.

A rationale behind the attempt to bind NTBI in the patients by administering apotransferrin is the prevention of iron-induced cytotoxicity, which is thought to depend on iron-catalysed formation of hydroxyl radicals (Halliwell & Gutteridge, 1990). NTBI is effectively taken up by parenchymal cells, particularly in the liver, and there are several lines of evidence suggesting that NTBI is toxic to liver cells (Anderson, 1999). On the other hand, NTBI in the sera of leukaemia patients has been shown to induce lipid peroxidation and it could thus damage cells even without uptake into the cells (Carmine *et al,* 1995). Ferric iron causes cytotoxicity after a few hours in liver cell cultures (Morel *et al,* 1990; Sakurai & Cederbaum, 1998) and it also rapidly impairs the phagocytic activity of polymorphonuclear leucocytes (van Asbeck *et al,* 1984). Thus, it appears that NTBI may damage various cell types. However, the role of NTBI, if any, in the severe toxic complications of myeloablative therapy is currently not known.

Another possible benefit of the binding of NTBI by apotransferrin could be the prevention of the growth of opportunistic bacteria and fungi. Practically all microorganisms are dependent on iron for growth. In normal plasma, transferrin keeps the level of free iron far too low to sustain the growth of microorganisms and only virulent bacterial species have developed mechanisms to acquire iron directly from transferrin (Ratledge & Dover, 2000). The increased level of free iron may thus predispose the neutropenic and immunosuppressed patients to septic infections by opportunistic bacteria and fungi that are dependent on NTBI for growth (Harrison *et al,* 1994; Iglesias-Osma *et al,* 1995; Matinaho *et al,* 2001). A further possible benefit could be that apotransferrin binds NTBI into a physiological form that can be utilized by the recovering bone marrow. As the erythroid progenitors take up a lot of transferrin-bound iron and, on the other hand, as the SCT patients typically have a low endogenous transferrin level (Sahlstedt *et al,* 2001), increase of the circulating transferrin pool by administering apotransferrin might promote the recovery of the graft. However, the therapeutic benefits of apotransferrin administration remain at this point merely speculative.

Patients 5 and 7, who remained negative for NTBI for the longest periods after the single apotransferrin dose, were both CML patients. We have previously found that during the peritransplantation period the endogenous transferrin levels in CML patients are significantly higher than in other patients (unpublished observation). This phenomenon may partly explain the better response to the apotransferrin dose in these patients. On the other hand, these two patients also had the lowest pretreatment serum ferritin levels. In this study, we found an inverse correlation between the pretreatment serum ferritin levels and the length of the NTBI-free period after the apotransferrin injection. However, as a result of the small number of patients in this study, it is not possible to make any far-reaching conclusions.

The present study demonstrated that, *in vivo*, the investigational apotransferrin product showed the biochemical effects that could be expected of functional human apotransferrin. After a single intravenous dose, the serum iron binding capacity was effectively, although in most patients temporarily, corrected and NTBI eliminated for variable periods. It remains to be shown whether the appearance of NTBI could be completely prevented by repeated administrations of apotransferrin and whether this would decrease the toxicity and opportunistic infections associated with myeloablative conditioning and stem cell transplantation.

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