Finnish Red Cross Blood Service and
Laboratory of Biochemistry and Microbiology,
Department of Chemical Technology,
Helsinki University of Technology, Finland

A PHARMACEUTICAL HUMAN APOTRANSFERRIN PRODUCT FOR IRON BINDING THERAPY

Leni von Bonsdorff

Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Department of Chemical Technology for public examination and debate in the Auditorium KE 2 (Komppa Auditorium) at Helsinki University of Technology (Espoo, Finland) on the 14th of November, 2003, at 12 noon.

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SUPERVISOR

Docent Jaakko Parkkinen, MD, PhD
Department for Research and Development
Finnish Red Cross Blood Service

REVIEWERS

Professor Maija Tenkanen, DrSc (Tech)
Department of Applied Chemistry and Microbiology
University of Helsinki
Helsinki, Finland

Docent Tapani Tuomi, DrSc (Tech)
Finnish Institute of Occupational Health
Helsinki, Finland

OPPONENT

Jan Over, PhD
Sanquin Plasma Products
Amsterdam, The Netherlands

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ABSTRACT

Transferrin is the major iron binding protein in human plasma. It binds iron with high affinity in a redox inactive form and delivers it to growing cells. Each molecule is capable of binding two molecules of ferric iron. Normally, transferrin is only about 30% saturated with iron. In certain clinical conditions, the iron concentration in serum is increased so that the iron binding capacity is exceeded and non-transferrin-bound iron (NTBI) is formed in serum. NTBI is potentially toxic because it generates free radical formation and can be taken up by tissues, leading to excess deposits that can potentiate tissue damage. It is also known that iron enhances the growth of bacteria and fungi, and can predispose patients to septic infections.

This thesis describes the development of an efficient process for producing pharmaceutical grade iron-free apotransferrin. The biochemical efficacy of apotransferrin for iron binding therapy was studied in early phase clinical trials in haematological stem cell transplant (SCT) patients. The scope of this work did not include studying the clinical efficacy of apotransferrin.

The manufacturing method used fraction IV of the Cohn cold ethanol human plasma fractionation process as starting material. Apotransferrin was purified in two ion exchange chromatography steps and ultrafiltration with over 90% recovery. In order to obtain a virus-safe product, the process comprised solvent detergent treatment as the main virus inactivation step and virus filtration and polyethylene glycol precipitation to remove physico-chemically resistant infectious agents. The purity of the product was at least 98%, main impurities being IgG, IgA and hemopexin. Methods for studying the iron binding capacity, the transferrin conformation and its iron forms, and the glycosylation variants were developed and used to study the quality of the finished product batches. The product had intact iron binding capacity and a native conformation. The results of several production batches indicated that the manufacturing could be carried out reproducibly. Product characterisation by electrospray and MALDI-TOF mass spectrometry indicated no other chemical modifications than N-linked glycan chains and disulphide bonds, except minor oxidation. A stable liquid formulation suitable for intravenous infusion was developed.

The biochemical binding of NTBI to apotransferrin *in vivo* was studied by several methods. The bleomycin method for NTBI determination was modified for microwell measurement and evaluated. The bleomycin assay was reproducible and NTBI was found in serum samples only when transferrin saturation was >80% and haemolysed samples were excluded. The bleomycin assay that measures redox-active iron underestimated the true concentration of NTBI. The concentration of NTBI could be calculated from the shift of transferrin iron forms found *in vivo* after intravenous

infusion of apotransferrin to patients. It could also be determined with a chelation based method, which, however, had a lower specificity than the bleomycin method. In haematological SCT patients, the concentration of NTBI could be as high as 20 µmol/l. Apotransferrin given in single intravenous doses to six patients bound NTBI effectively, although in most cases temporarily. With repeated high dose regimens, the appearance of NTBI was prevented in 5 of 8 patients.

The influence of NTBI on the growth of the opportunistic pathogen *Staphylococcus epidermidis* was studied both with purified transferrin and in serum milieu. In both cases, growth was critically dependent on NTBI and on a high transferrin saturation. Only at high initial bacterial concentrations could growth be detected with partially saturated transferrin. Apotransferrin administered to SCT patients bound NTBI and restored the growth inhibitory effect of serum. Exogenous apotransferrin might protect the patients against infections by *S. epidermidis* and other opportunistic pathogens whose growth is dependent on NTBI.

In conclusion, the apotransferrin was pure and safe and showed *in vivo* the biochemical effects that could be expected of a functional human apotransferrin product. In SCT patients it was possible to prevent the appearance of NTBI and maintain the bacterial growth inhibitory effect in serum.

PREFACE

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Helsinki, October 2003

Leni von Bonsdorff

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by their Roman numerals.

I **von Bonsdorff L**, Tölö H, Lindeberg E, Nyman T, Harju A, Parkkinen J. Development of a pharmaceutical apotransferrin product for iron binding therapy. Biologicals 2001;29:27-37.

II **von Bonsdorff L**, Lindeberg E, Sahlstedt L, Lehto J, Parkkinen J. Bleomycin-detectable iron assay for non-transferrin-bound iron in hematologic malignancies. Clin Chem 2002;48:307-314.

III Sahlstedt L, **von Bonsdorff L**, Ebeling F, Ruutu T, Parkkinen J. Effective binding of free iron by a single intravenous dose of human apotransferrin in haematological stem cell transplant patients. Br J Haematol 2002;119:547-553.

IV Matinaho S, **von Bonsdorff L**, Rouhiainen A, Lönnroth M, Parkkinen J. Dependence of *Staphylococcus epidermidis* on non-transferrin-bound iron for growth. FEMS Microbiol Lett 2001;196:177-182.

V **von Bonsdorff L**, Sahlstedt L, Ebeling F, Ruutu T, Parkkinen J. Apotransferrin administration prevents growth of *Staphylococcus epidermidis* in serum of stem cell transplant patients by binding of free iron. FEMS Immunol Med Microbiol 2003;37:45-51.

In publication III, the authors L. Sahlstedt and L. von Bonsdorff contributed equally.

ABBREVIATIONS

AAS	Atomic absorption spectrophotometry
ATCC	American Type Culture Collection
BPT	Bathophenantroline
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CFU	Colony forming units
CRP	C-reactive protein
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
FeNTA	Ferric nitrilotriacetate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAV	Hepatitis A virus
HIV	Human immunodeficiency virus
IEX-HPLC	Ion exchange high performance liquid chromatography
IgA	Immunoglobulin A
IgG	Immunoglobulin G
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
NTA	Nitrilotriacetic acid
NTBI	Non-transferrin-bound iron
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RP-HPLC	Reversed phase high performance liquid chromatography
SCT	Stem cell transplant
SD	Solvent detergent
SDS	Sodium dodecyl sulphate
SEC-HPLC	Size exclusion high performance liquid chromatography
TBA	Thiobarbituric acid
TBE	Tris-borate-EDTA

INTRODUCTION

1 Transferrin and serum iron

1.1 Transferrin characteristics

Transferrins represent a class of proteins found in biological fluids of all vertebrates and invertebrates with the property of reversibly binding iron. The name transferrin was suggested by Holmberg and Laurell (1947) and is used as the name for the iron-binding β-globulin found in blood serum and other extracellular fluids of vertebrates (de Jong *et al.*, 1990). Other proteins of the same group are lactoferrin in milk and in neutrophil granules (Metz-Boutigue *et al.*, 1984), ovotransferrin of egg white and melanotransferrin (antigen p97) produced by melanoma cells (Brown *et al.*, 1982). Transferrin was first detected and isolated in works described by Holmberg and Laurell (1945) and Schade and Caroline (1946). The latter group used the newly developed Cohn fractionation procedure to prepare the iron-binding fraction of plasma. Since then, the protein has been thoroughly studied and characterised (de Jong *et al.*, 1990; Evans *et al.*, 1999; Harris and Aisen, 1989).

Human transferrin is a single polypeptide chain containing 679 amino acid residues (MacGillivray *et al.*, 1983). The protein has 19 disulphide bridges. Crystallographic studies have shown that the transferrin molecule is organised into two homologous lobes of about 330 amino acid residues, the N- and the C-lobe. The lobes are linked by a short flexible spacer peptide and each lobe contains two dissimilar domains divided by a cleft which is the binding site for Fe^{3+} (Bailey *et al.*, 1988; Wang *et al.*, 1992) (Figure 1). At the iron binding site, four of the six Fe^{3+} co-ordination sites are occupied by the protein ligands (2 tyrosine, 1 histidine and 1 aspartate residue) and two by the bidentate carbonate anion (Bailey *et al.*, 1988; Hirose, 2000). The synergistic binding of an anion, preferentially the carbonate molecule, is essential for the iron binding (Harris and Aisen, 1989).

Two N-linked oligosaccharides are found in the C-lobe at aspargine residues Asn^{413} and Asn^{611}. The carbohydrates constitute about 6% of the mass of transferrin that has a molecular weight of 79750. The glycan chains are mainly biantennary (85%) and triantennary (15%) complex-type glycans (Fu and van Halbeek, 1992; Spik *et al.*, 1985). The number of sialic acid residues per transferrin molecule is between 4 and 6. Glycosylation variants occur in different conditions. In patients suffering from alcoholism, carbohydrates lacking two to four of the terminal trisaccharides, comprising the negatively charged sialic acid and the neutral *N*-acetylglucosamine and galactose have been found, and disialotransferrin and to a lesser degree mono- and

asialotransferrin are present (Stibler, 1991). Such carbohydrate-deficient transferrin can be used as a marker for alcoholism (Turpeinen *et al.*, 2001). Increase of branching has been found during pregnancy (Leger *et al.*, 1989) and in congenital glycosylation disorder, when there also is an increase in fucosylation (Mills *et al.*, 2001; Yamashita *et al.*, 1993). The variation of glycosylation is a determinant of the microheterogeneity of transferrin. Another determinant is the genetic polymorphism. Genetic variants were first detected by starch electrophoresis. The most common was designated TfC, the more anodal TfB and cathodal TfD. Variants within the subgroups have been identified with isoelectric focusing (Kamboh and Ferrell, 1987). Of at least 38 variants, only 4 occur with a frequency over 1% (de Jong *et al.*, 1990). Among Caucasians, the C variant, having several subtypes including C1, C2 and C3, is found almost exclusively (Tenkanen *et al.*, 1989). A single amino acid substitution determines the TfC1 and TfC2 variants (Namekata *et al.*, 1997). The highest frequencies of the D variant is found among African blacks, although hardly ever as homozygous D phenotypes (Kasvosve *et al.*, 2000). Attempts to establish a relationship between the transferrin variant, serum transferrin concentration, and iron-binding capacity in Caucasians have been inconclusive (Sikstrom *et al.*, 1993). A functional difference was demonstrated between CD and CC phenotypes in blacks, with a lower *in vitro* binding of iron to transferrin for the CD individuals and differences in serum iron parameters between the phenotypes (Kasvosve *et al.*, 2000). In a transferrin variant where an amino acid substitution in the C-lobe leads to an open structure of the iron saturated molecule, a reduced affinity for the transferrin receptor was found (Evans *et al.*, 1994b).

Each molecule of transferrin can bind two Fe^{3+} ions. The four iron forms of transferrin are the iron-free apotransferrin, the monoferric transferrins with iron in the C- or the N-lobe, respectively, and the diferric holotransferrin. The affinity for iron at physiological pH 7.4 is high, with a binding constant of about 10^{22} (Harris and Aisen, 1989). Upon binding of iron, the lobes undergo a conformational transition from the apo-structure with an open interdomain cleft to a closed holo-structure (Hirose, 2000). The conformational change can be studied by urea polyacrylamide gel electrophoresis first described by (Makey and Seal, 1976), whereby the different iron forms of transferrin can be resolved. The presence of urea causes the lobes that do not contain iron to unfold and decreases their mobility (Evans and Williams, 1980). It is believed that the change in conformation is important for the binding of the molecule to the transferrin receptor, and diferric transferrin has a 10-100 times higher affinity for the receptor than apotransferrin (Richardson and Ponka, 1997). The binding mechanism of transferrin to its receptor is not fully elucidated, but it is believed that the recognition site requires the presence of both transferrin lobes (Mason *et al.*, 1997; Mason *et al.*, 2002; Zak *et al.*, 2002) and that the glycans are of less importance (Hoefkens *et al.*, 1997). In circulation, where normally only a third of the transferrin iron binding sites are occupied, iron preferentially binds to the N-terminal lobe which is also called the acid labile lobe

(Evans *et al.*, 1999). The metal release from transferrin is mainly accomplished by a decrease in pH, where protonation of the iron ligands causes release of the iron. The iron release can also be accelerated by other chemical compounds capable of complexing iron such as pyrophosphates (Morgan, 1979) and citrate (Gumerov *et al.*, 2003). Transferrin is capable of binding several other metals, but with a lower affinity (Harris and Aisen, 1989).

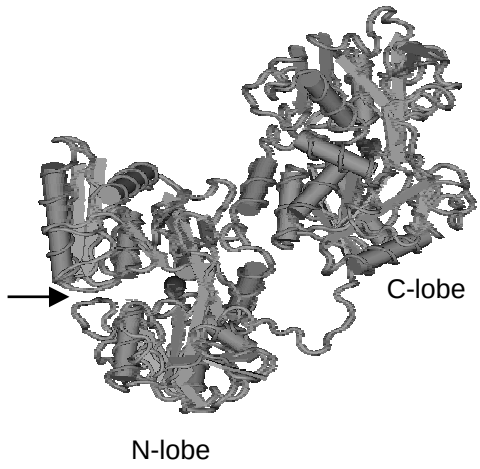

Figure 1. A ribbon diagram of a diferric rabbit serum transferrin molecule. The arrow indicates the position of the Fe^{3+} molecule in the inter-domain cleft in the N-lobe. The model is based on the PDB atomic coordinate file 1JNF (Hall *et al.*, 2002).

1.2 Iron and role of transferrin

Iron is essential for virtually all living organisms. It is needed in proteins for oxygen transportation and for enzymes that can catalyse redox reactions and DNA synthesis. In biological systems and aqueous solutions, the transition metal iron can exist in two oxidative states as ferrous (Fe^{2+}) or ferric iron (Fe^{3+}), capable of respectively donating or accepting electrons. In aerobic conditions, Fe^{2+} is oxidised to Fe^{3+}, which is the most common form found in the environment. The properties of iron are associated with two types of problems. First, Fe^{3+} is highly insoluble at physiological pH. With increasing pH, Fe^{3+} readily hydrolyses to hydroxy-forms, followed by irreversible formation of hydroxy-iron polymers that precipitate. At physiological pH 7, the solubility of Fe^{3+} is only about 10^{-18} mol/l (Spiro and Saltman, 1974). Secondly, the redox active iron can be

toxic as it is capable of forming harmful free radicals (Halliwell and Gutteridge, 1985). Ferrous iron catalyses the formation of the highly oxidising hydroxyl radical (OH·) from hydrogen peroxide, known as the Fenton reaction (1). Ferric iron can be reduced to ferrous iron by superoxide (O_2^-). This net reaction (2) is referred to as the Haber-Weiss reaction. However, the term "Fenton-catalysed Haber-Weiss reaction" often used to describe these reactions, has been criticised. *In vivo*, the superoxide is mainly considered to be the source of hydrogen peroxide for the Fenton reaction, and other agents as well as superoxide can reduce iron. Toxicity therefore arises from the Fenton reaction alone, and the Haber-Weiss reaction probably does not take place (Koppenol, 2001).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot \qquad (1)$$

$$O_2^- + H^+ + H_2O_2 \rightarrow O_2 + OH\cdot + H_2O \qquad (2)$$

The hydroxyl radical is, although short-lived, highly reactive and able to cause secondary radicals and initiate the lipid peroxidation chain reaction. It also causes DNA strand breaks, inactivates enzymes and can depolymerise polysaccharides (McCord, 1998).

In the blood stream, iron is transported to the cells bound to transferrin. This type of iron transportation brings several benefits. First, the transferrin is only about 30% saturated with iron to ensure capacity to sequester available iron. Second, iron is kept in a redox-inactive form. It has been shown that transferrin-bound iron is not capable of promoting hydroxyl radical formation (Baldwin *et al.*, 1984). Third, iron is not hydrolysed as it would be if were in the form of a free salt and can be targeted directly to cells requiring iron. Transferrin-bound iron is taken up by the cells by receptor-mediated endocytosis (Richardson and Ponka, 1997) whereafter apotransferrin is re-circulated to the blood stream.

Iron is used for the synthesis of proteins requiring iron for biological activity, or stored in intracellular ferritin. Several other iron-binding proteins are involved in the human iron metabolism. Although transferrin is of utmost importance in the transportation of iron, the ~3-4 mg of transferrin-bound iron is only ~0.1% of the total body iron of ~4000 mg. However, the transferrin iron turnover is significant and about 30 mg of iron is transported daily to the cells. Of this, about 80% is transported to the bone marrow for haemoglobin synthesis in developing erythroid cells. Haemoglobin iron comprises 60-70% of body iron, followed by the intracellular storage protein ferritin that holds 10-20% of iron. The rest of the iron is found in myoglobin, cytochromes and iron-containing enzymes. Body iron stores are highly conserved, only 1-2 mg is lost daily

and the iron balance is maintained by dietary iron absorption (Conrad and Umbreit, 2000; Fairbanks and Beutler, 1995).

1.3 Serum non-transferrin-bound iron (NTBI)

In certain clinical conditions, the iron concentration in serum is increased. If the iron binding capacity of transferrin is exceeded, non-transferrin-bound iron (NTBI) can form in serum. The reasons for the increase of iron are diverse (Table 1). Although the causes of NTBI vary, it is generally considered that the presence of NTBI is a pathological manifestation and is never found in healthy individuals (Breuer *et al.*, 2000a). NTBI was first detected in chronic iron overload disorders such as haemochromatosis. It can also be detected in acute events such as for haematological patients undergoing myeloablative conditioning therapy, and especially those who receive stem cell transplants (SCT) (Bradley *et al.*, 1997; Sahlstedt *et al.*, 2001). The myeloablative conditioning therapy results in a halt of erythropoiesis, the only effective way of transferrin iron utilisation. If iron release to circulation continues from macrophages taking up senescent erythrocytes, this results in a remarkable increase in the total serum iron level.

The form and nature in which NTBI exists in serum is not fully elucidated (Esposito *et al.*, 2002; Hider, 2002). Primarily, the formation of NTBI was considered a spillover phenomenon when the transferrin saturation was exceeded. According to (Grootveld *et al.*, 1989), a significant portion of NTBI in haemochromatosis is in the form of citrate and, possibly, acetate complexes. The tendency of iron to form large iron-polymers at physiological conditions could lead to different types of iron-citrate complexes, varying in size from monomeric to large oligomer complexes with even 17-19 iron molecules (Hider, 2002). Albumin could also act as an iron binding ligand due to the presence of large number of negatively charged carboxylate sites (He and Carter, 1992). In a study using pure albumin, it was found that albumin had capacity to bind added iron which resulted in inhibition of lipid peroxidation (Loban *et al.*, 1997). Studies with hypotransferrinaemic mice have indicated a mixture of iron species with differing reactivity to NTBI assays (Simpson *et al.*, 1992). The concentrations varied in the range of 1-20 µmol/l depending on the assay used to measure NTBI. No detectable mononuclear iron was measured by electron paramagnetic resonance spectrometry, indicating lack of citrate-bound iron in monomeric form. Thus, NTBI is probably a mixture of different forms of iron bound to extracellular components, and the proportions of the iron forms may vary between patient groups and clinical conditions.

NTBI is potentially toxic because it generates free radical formation and can be taken up

Table 1. Examples of clinical conditions when NTBI has been detected in serum.

Clinical condition	Suggested mechanism for formation of NTBI	Reference
Haemochromatosis	Increased iron absorption from the diet leads to iron accumulation and overload	(Grootveld *et al.*, 1989)
Secondary iron overload disorders (including thalassemia)	Multiple: Ineffective erythropoiesis, red cell transfusions, increased intake or absorption of iron	(Bottomley, 1998; Hershko *et al.*, 1978)
Chemotherapy of haematologic malignancies	Drug-induced halt of erythropoiesis and strongly decreased uptake of transferrin iron	(Bradley *et al.*, 1997; Carmine *et al.*, 1995; Harrison *et al.*, 1994; Sahlstedt *et al.*, 2001)
Premature and full-term neonates	Ferro-oxidase deficiency, low transferrin concentration, accelerated erythrocyte turnover	(Evans *et al.*, 1992); (Lindeman *et al.*, 1992); (Lindeman *et al.*, 2000)
Cardiopulmonary bypass	Haemolysis	(Pepper *et al.*, 1995)
Haemodialysis patients	Intravenous iron infusion	(Breuer *et al.*, 2000b; Parkkinen *et al.*, 2000)
Fulminant liver failure	Possible release of iron from damaged liver cells	(Evans *et al.*, 1994a)
Atransferrinemia	Low levels of transferrin	(Simpson *et al.*, 1992)* (Beutler *et al.*, 2000; Craven *et al.*, 1987; Hamill *et al.*, 1991)

*NTBI detected in hypotransferrinaemic mice

by tissues, leading to excess deposits that can potentiate tissue damage. NTBI is taken up by cells by a transferrin independent transport system which is rapid, but not regulated in the same manner as the transferrin receptor system and may involve a lipocalin transport mechanism (Kaplan, 2002). It is mainly taken up by parenchymal cells (Craven *et al.*, 1987), particularly in the liver (Brissot *et al.*, 1985) and there are several lines of evidence suggesting that NTBI is toxic to liver cells (Anderson, 1999). Ferric iron causes cytotoxicity already after a few hours in liver cell cultures (Morel *et al.*, 1990; Sakurai and Cederbaum, 1998) and it also rapidly impairs the phagocytic activity of polymorphonuclear leukocytes (van Asbeck *et al.*, 1984). Most of the toxic effects of the redox-active NTBI are believed to take place intracellularly, when iron accumulates and the cellular defence mechanism is overwhelmed. On the other hand, sera of leukaemia patients has been shown to induce lipid peroxidation. NTBI could thus damage cells even without uptake into the cells (Carmine *et al.*, 1995). Because it appears that NTBI may damage various cell types, it is possible that NTBI plays a part

in the pathogenesis of mucosal and liver injury associated with high-dose chemotherapy (Beare and Steward, 1996; Carmine *et al.*, 1995).

1.4 Measurement of NTBI in serum

A number of methods have been developed to measure NTBI in serum. A prerequisite for the determination is to avoid interference of iron bound in redox-inactive form to transferrin or ferritin, or to therapeutic chelating agents such as desferrioxamine. Hershko *et al.* (1978) were the first to describe a method to measure NTBI, where the iron was mobilised with a chelating agent (EDTA) and after ultrafiltration, the chelated iron complex in the ultrafiltrate was measured. Several methods based on a similar chelation principle before quantitation have been described (Table 2). Nitrilotriacetate (NTA) is mostly used for iron chelation (Figure 2).

In the bleomycin-detectable iron assay iron is not chelated before determination, but is bound in its ferrous form to the glycopeptide antibiotic bleomycin (Figure 2). Ascorbic acid is added as a reduction agent. The formed iron-bleomycin undergoes complex autooxidation which results in the release of the reactive hydroxyl radical (Sugiura *et al.*, 1982). The hydroxyl radicals damage added DNA and results in the release of malondialdehyde. The amount of bleomycin-iron is proportional to the degree of DNA degradation (Evans and Halliwell, 1994). Malondialdehyde, formed from deoxyribose residues of DNA, can be measured by the thiobarbituric acid test (Gutteridge *et al.*, 1981), which is the most widely used quantitation method. An alternative quantitation method has been described by Burkitt *et al.* (2001) where an ethidium-binding assay was used to measure DNA degradation. The bleomycin assay has been compared with another biochemical assay based on the activation of the iron free aconitase enzyme in presence of NTBI (Mumby *et al.*, 1998). Both biochemical methods have given similar results. The bleomycin-detectable iron assay has been used to study the occurrence of NTBI in various clinical conditions and biological fluids (Halliwell and Gutteridge, 1990).

In patients with haematologic malignancies, NTBI has been studied both with the bleomycin assay and with the chelation method. The reported NTBI concentrations vary depending on the method for determination, and it is difficult to conclude the true concentration of NTBI based on these results. The highest reported bleomycin-detectable iron concentrations of 21.5 and 28 µmol/l were found by Halliwell *et al.* (1988) and Harrison *et al.* (1994), respectively. In these studies, an early version of the bleomycin method was used (Gutteridge *et al.*, 1981). In the early version, the TBA-chromogen was measured directly in the reagent mixture and not extracted into butanol. It is possible that interfering compounds remained in the mixture. Carmine *et al.* (1995)

measured much lower concentrations in the range of 0.1 – 1 µmol with the bleomycin assay. Bradley *et al.* (1997) used the chelation method with NTA and HPLC quantitation of the chelated iron-complex that gave NTBI values up to 5 µmol/l. Similar values (2-7 µmol/l) were obtained by Durken *et al.* (1997) when NTA-complexed iron was quantified by atomic absorption spectrophotometry.

Table 2. Assays for measurement of serum NTBI

Assay type	Quantitation of iron	Reference
1. Chelation of NTBI		
Chelation of NTBI with EDTA, ultrafiltration of complex	Colorimetric	(Hershko *et al.*, 1978)
Chelation of NTBI with NTA, ultrafiltration of complex	HPLC	(Porter *et al.*, 1996; Singh *et al.*, 1990)
	Colorimetric using bathophenanthroline chromogen	(Gosriwatana *et al.*, 1999; Zhang *et al.*, 1995)
	Atomic absorption spectrophotometry	(Durken *et al.*, 1997; Gosriwatana *et al.*, 1999; Jakeman *et al.*, 2001)
Chelation of NTBI with oxalate, transfer of oxalate to immobilised desferrioxamine	Fluorescence using metallosensor calcein	(Breuer *et al.*, 2000b)
Chelation of NTBI with desferrioxamine	Fluorescence quenching of fluorescein-desferrioxamine probe	(Breuer *et al.*, 2001; Breuer and Cabantchik, 2001)
2. Biochemical reactions caused by NTBI		
Bleomycin assay. Ferrous iron complexed with bleomycin causes DNA degradation, degradation products are measured.	Thiobarbituric acid reaction, spectrophotometry	(Evans and Halliwell, 1994; Gutteridge *et al.*, 1981; Gutteridge and Hou, 1986)
	Ethidium-binding assay	(Burkitt *et al.*, 2001)
Activation of iron-sulphur cluster of aconitase	Enzymatic	(Mumby *et al.*, 1998)

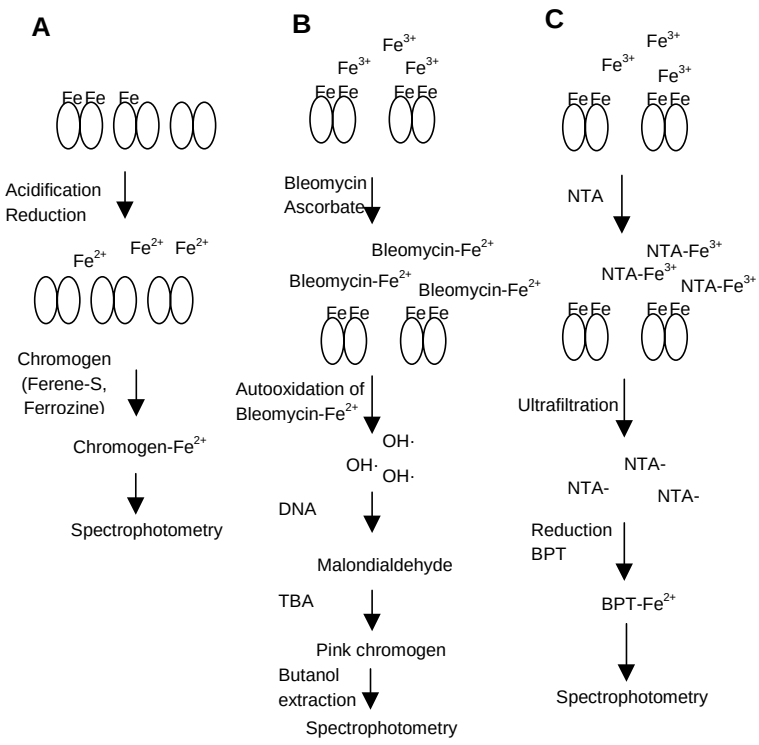

Figure 2. A schematic representation comparing a serum transferrin iron and two NTBI assay methods. The double circles represent the transferrin molecule with two lobes, each capable of binding one iron molecule.

A, A chromogenic method measuring transferrin-bound iron. The iron is released from transferrin by acidification and reduced. Ferrous iron reacts with the chromogenic agent which can be either Ferene-S or Ferrozine and the coloured complex is measured by spectrophotometry.

B, The bleomycin assay measuring NTBI. The bleomycin reacts with NTBI in the sample after reduction using ascorbate. The bleomycin-iron complex undergoes complex autooxidation which results in the release of the reactive hydroxyl radical (OH·). The hydroxyl radicals damages the added DNA and results in the release of malondialdehyde. This reacts with thiobarbituric acid (TBA) and forms a pink chromogen, which after extraction into butanol is measured by spectrophotometry.

C, The chelation assay measuring NTBI. The chelator nitrilotriacetate (NTA) is added to the sample to chelate NTBI. The chelated iron is separated from transferrin and other serum proteins by ultrafiltration. The iron is complexed after reduction using bathophenantroline, and measured by spectrophotometry.

2 Iron and infection

2.1 NTBI, microbial growth, and risk of infection

Like all living organisms, microbes are dependent on iron for growth, with the exception of some lactic acid bacteria (Archibald, 1983). The low availability of iron *in vivo* restricts the growth of invading bacteria. This mechanism, often described as the iron withholding system (Weinberg, 1984), is important in the defence against infections. It was first demonstrated by Schade and Caroline (1944), who showed that raw hen egg white inhibited the growth of several bacterial strains and that this inhibition could be overcome by addition of iron. Later this effect was ascribed to the ovotransferrin in egg white, and a similar mechanism was found in serum (Schade and Caroline, 1946).

To overcome the iron-limiting conditions, pathogenic bacteria have evolved specific high affinity iron-uptake mechanisms to acquire iron from the host. Pathogenic neisseriae and *Haemophilus influenza* (Gray-Owen and Schryvers, 1996; Schryvers and Stojiljkovic, 1999) possess surface receptors that directly bind transferrin. Receptors that specifically bind lactoferrin, haemoglobin, or haem have also been found (Wandersman and Stojiljkovic, 2000). Another mechanism whereby bacteria and fungi can acquire iron from the host is through synthesis and excretion of low-molecular-weight compounds that chelate iron with high affinity; these are known as siderophores. Siderophores are produced by many, both pathogenic and non-pathogenic micro-organisms, in response to a deficiency of iron and may even sequester iron attached to molecules such as transferrin, lactoferrin, and ferritin. The sequestered iron is taken up via siderophore receptors on the cells. Well characterised siderophore systems are found in enteric gram-negative bacteria Escherichia, Klebsiella, and Salmonella (Griffiths and Williams, 1999). Siderophores are also produced by gram-positive bacteria; e.g. *Staphylococcus aureus* (Trivier and Courcol, 1996) and pathogenic fungi (Howard, 1999). The ability to produce siderophores *in vitro* is not, however, necessarily associated with an increased virulence of the bacteria (Griffiths and Williams, 1999; Ratledge and Dover, 2000). The siderophore may not be able to effectively dissociate iron from the iron-binding proteins, or the iron-siderophore complex may not necessarily be transported back to the organism. However, for some bacterium as for the opportunistic pathogen *Pseudomona aeruginosa*, it has been shown *in vivo* through an animal model that the siderophore pyoverdin was essential for the virulence (Meyer *et al.*, 1996).

The virulence of many organisms has been considerably enhanced in experimental infections by injecting iron compounds into an animal host (Griffiths, 1999). Clinical conditions with iron overload have been associated with an increased risk of infection.

Vibrio vulnificus is an example of an opportunistic bacterium that causes infections in patients with haemochromatosis (Bullen *et al.*, 1991). A risk for *Yersinia enterocolita* septicaemia is clearly associated with iron overload disorders, especially when desferrioxamine is used for iron chelation (Bottone, 1997; Mofenson *et al.*, 1987). Lambert and Hunter (1990) suggested that high transferrin saturation might contribute to lethality of pneumococcal pneumonia.

Haematological patients receiving high-dose chemotherapy and stem cell transplantation have an increased risk for infections which can be life-threatening. In addition to neutropenia as the major host defence defect, the use of central venous catheters, severe mucositis and increased rates of antibiotic resistant pathogens are recognised risk factors (Ehni *et al.*, 1991; Elishoov *et al.*, 1998; Gonzalez-Barca *et al.*, 1996). Hospital-derived gram-positive infections have become more common during the last two decades (Zinner, 1999), predominated by coagulase negative staphylococci (Wade *et al.*, 1982; Winston *et al.*, 1983). Also fungal infections (candidiasis and aspergillosis) are common (Jantunen *et al.*, 1997; Morrison *et al.*, 1993). It has been speculated that the increased rate of infections in this patient group also could be related to the high serum iron concentrations with highly saturated transferrin. Caroline *et al.* (1969) proposed that high transferrin saturation predisposed patients with acute leukemia to infections by the yeast *Candida albicans*. An association between the high transferrin saturation and bacterial growth was described by Hunter *et al.* (1984), who showed that serum from leukemic patients had a reduced ability to inhibit the growth of *Pseudomonas aeruginosa*. A similar *in vitro* effect was found for *Escherichia coli* and *Staphylococcus aureus* (Gordeuk *et al.*, 1986). High transferrin saturation level was proposed as a possible risk factor for systemic fungal infections in patients with haematological malignancies (Iglesias-Osma *et al.*, 1995; Karp and Merz, 1986).

The presence of NTBI may therefore be a contributing virulence factor to opportunistic bacteria and fungi, and may predispose these immunocompromised patients to infections and septicaemia (Ward and Bullen, 1999). Binding of NTBI into a redox-inactive form could possibly decrease the high infection risk in the patients. Several studies have shown that transferrin displays a growth inhibitory effect *in vitro* on opportunistic organisms, such as *Vibrio vulnificus* (Chart and Griffiths, 1985), *Rhizopus* (Boelaert *et al.*, 1993), *Candida albicans* (Caroline *et al.*, 1969; Shiraishi and Arai, 1979) and *Histoplasma capsulatum* (Sutcliffe *et al.*, 1980). Recently, an anticryptococcal protein in serum was identified as apotransferrin (Sridhar *et al.*, 2000). Iron chelating agents, such as the most widely used intravenous desferrioxamine and the recently licensed oral deferiprone, have not been used in this patient group (Porter, 2001; Weinberg, 1999). Desferrioxamine is a siderophore produced by *Streptomyces pilosus*, and it is used for iron chelation therapy of patients with iron overload or acute iron poisoning. The use of desferrioxamine can increase the risk of infections of bacteria

or fungi that are capable of utilising this siderophore for growth, e.g. bacteremia by *Yersinia enterocolita* (Bottone, 1997) or fungal mucormycosis caused by *Rhizopus* (Boelaert *et al.*, 1993). Desferrioxamine is also known to have dose related toxicity (Porter and Huehns, 1989).

2.2 Iron acquisition of *Staphylococcus epidermidis*

The coagulase negative staphylococcus *S. epidermidis* is a gram-positive bacterium in normal skin flora. It is an opportunistic pathogen, and is increasingly found in hospital-derived blood-stream infections. The wide-spread use of intravascular catheters in seriously ill and immunocompromised patients, and selection of antibiotic-resistant strains are factors which have contributed to the increased infection rates (Kloos and Bannerman, 1994).

The iron acquisition by staphylococci from transferrin has been studied *in vitro* starting with the work of Schade (1963), who found that coagulase positive *S. aureus* was able to proliferate in normal serum, whereas coagulase negative staphylococci (*S. albus*) could not grow in serum without iron supplementation. This suggested that the *S. aureus* strains could aquire iron from partially saturated transferrin whereas the other strains could not. Iron uptake studies using human transferrin labelled with iron isotope showed that *S. aureus* acquired iron from transferrin whereas *S. epidermidis* did not (Lindsay *et al.*, 1995). The staphylococci have been described to produce at least three types of siderophores (Trivier and Courcol, 1996) of which staphyloferrin A was shown to sequester iron from diferric transferrin when grown *in vitro* (Modun *et al.*, 1998). In addition to a siderophore mediated uptake mechanism, a bacterial cell-surface protein, identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was described to act as a transferrin receptor for *S. aureus* and *S. epidermidis* (Modun *et al.*, 1998; Modun *et al.*, 2000). *S. epidermidis* grew in the presence of diferric transferrin and converted transferrin into the iron free form at the high bacterial concentrations (10^8 CFU/ml) used in the study (Modun *et al.*, 1998).

3 Methods to produce apotransferrin

Transferrin has been isolated and purified making use of a wide variety of starting materials and purification methods in laboratory scale preparation. Methods that are suitable for large scale manufacturing of transferrin have mainly been developed with the aim to produce transferrin for use in research and in bulk quantities for cell culture media. Many methods are based on the use of fraction IV from the Cohn cold ethanol fractionation process for human plasma (Cohn *et al.*, 1946) as starting material, and

employ ion exchange chromatography for protein purification. Inman *et al.* (1961) used the Cohn fraction IV and purified transferrin by use of precipitation and batch ion exchange adsorption techniques to prepare ≥90% pure, iron-saturated transferrin with a yield of about 40%. The method of Kistler *et al.* (1960) comprised a combination of ethanol and rivanol precipitation to obtain about 85% pure transferrin, followed by crystallisation to increase purity to 99%. Later methods include specific steps to inactivate or remove blood-born viruses. β-propiolactone/UV-irradiation was employed to inactivate viruses after purification by precipitation in a method by Bethke *et al.* (1991). Haupt (1993) used pasteurisation in combination with a complexing agent, either citrate or EDTA, to prepare virus safe apotransferrin. Solvent detergent treatment in combination with virus removal filtration was used in a method for preparation of iron-saturated transferrin (Rolf *et al.*, 1997). Rivat *et al.* (1992) described a single-step method to produce transferrin with 97% purity and 75% yield from a by-product of a chromatographic plasma fractionation method that included virus inactivation using the solvent detergent treatment method (Horowitz *et al.*, 1985).

Recombinant transferrin molecules have been produced in small laboratory scale. These transferrin products have mainly been used in structural and functional protein analysis. Full length human transferrin has been produced in BHK cells (Mason *et al.*, 1993) and in a baculovirus system (Ali *et al.*, 1996). Recombinant N- and C-terminal half molecules have been expressed in both eukaryotic expression systems (Funk *et al.*, 1990; Mason *et al.*, 1996; Mason *et al.*, 1997; Mason *et al.*, 2001; Zak and Aisen, 2002) and prokaryotic systems (Hoefkens *et al.*, 1996).

The transferrin products obtained by the described methods vary with respect to iron content, protein purity and degree of polymerisation and aggregation, and the yield of product is often low. For use as a therapeutic agent in iron binding therapy, an apotransferrin product needs to have a native conformation, it has to be in the iron-free form, and, it must be safe for intravenous administration. At the same time, the production method has to be cost effective to enable production of large, up to kilogram, product quantities .

AIMS OF THE STUDY

The general objectives were to develop an apotransferrin preparation and study its biochemical efficacy in iron binding therapy. The specific objectives were:

- to develop an efficient process for manufacturing of virus-safe pharmaceutical apotransferrin

- to characterise the apotransferrin product

- to evaluate methods for NTBI determination in serum

- to study the binding of NTBI in serum by apotransferrin administration

- to study the dependence of the growth of *Staphylococcus epidermidis* on NTBI, and the effect of apotransferrin on the growth of *S. epidermidis* in patient serum

MATERIALS AND METHODS

The methods are described in detail in the original publications (I-V) and briefly below.

1 Methods for production, identification and characterisation of apotransferrin (I)

1.1 Apotransferrin production method

The production of apotransferrin described in publication I was carried out as follows: Apotransferrin was purified from fraction IV of the Cohn cold ethanol fractionation method for human plasma, based on the Krijnen modification of Cohn method 6 (Kistler and Friedli, 1980; Krijnen, 1970). The fraction IV paste was dissolved in water, 5% PEG was added, and the protein precipitate was removed by depth filtration to obtain a clarified crude transferrin solution. Cation exchange chromatography using SP Sepharose was used to capture the proteins, and the transferrin fraction was eluted using a pH elution. The partially purified transferrin solution was subjected to solvent detergent (SD) treatment to inactivate enveloped viruses. The main purification step was the second chromatographic step using Q Sepharose, that also removed the SD-reagents. The product was diafiltered, and finally virus-filtered using a 15 nm filter. It was formulated to a 50 g/l protein concentration in a 150 mmol/l sodium chloride solution at pH 6. The product was sterile filtered and aseptically filled into bottles and stored as a liquid solution at 2-8 °C or optionally lyophilised. The process flow scheme is shown in Fig. 3.

1.2 Identification of transferrin

N-terminal sequencing was used for identification of apotransferrin fragments (Matsudaira, 1987). Peptides for mass mapping were prepared by proteolytic digestions with endoproteinase LysC on native and deglycosylated apotransferrin after reduction and alkylation, and desalting by RP-HPLC. The molecular mass of the peptides was determined by MALDI-TOF mass spectrometry (Vorm *et al.*, 1994).

1.3 Apotransferrin characteristics and purity

The methods used to determine the product characteristics and purity are described in detail in publication I. Methods especially set up to study transferrin are described here.

Determination of transferrin iron forms

Urea-polyacrylamide gel electrophoresis (urea-PAGE) for determination of the iron forms of transferrin was carried out according to an adaptation by Williams *et al.* (1978) of the original method of Makey and Seal (1976). Pure transferrin samples of 15-20 µg were separated in 6% gels (10x10cm) with 6 mol/l urea for 2 h at 170 V constant voltage. Gels were prepared and run with TBE buffer (0.1 mol/l TRIS, 0.01 mol/l boric acid, 0.0016 mol/l EDTA, pH 8.4). The gels were stained with Coomassie Brilliant Blue.

Determination of the iron binding capacity

The iron binding capacity of transferrin was determined by titration with ferric nitrilotriacetic acid (Welch and Skinner, 1989). 100 µl aliquots of ferric nitrilotriacetate solution (1-1000 µmol/l) were added to 900 µl samples of apotransferrin (3.3 g/l) in 0.3 mol/l Tris-HCl, pH 8, to give final concentrations of ferric nitrilotriacetate ranging from 0 to 100 µmol/l. After incubation for 1 h at 20-25°C, samples of 350 µl were transferred to a microtiter plate and the absorbance was measured at 450 nm using a microplate spectrophotometric reader (Titertek Multiscan RC, Labsystems). The amount of iron required for full saturation was determined from the inflection point of the absorbance plot, and the iron binding capacity (%) was calculated by dividing this amount with the theoretical amount of iron required to fully saturate the added transferrin. The molar concentration of transferrin was calculated using a molecular mass of 79570 for transferrin (Morgan, 1992).

Identification of transferrin glycosylation variants

A high resolution ion exchange chromatography method was used to separate glycosylation variants of transferrin. Samples were injected on to a Mono Q HR 5/5 column (Amersham Biosciences). The equilibration buffer was 0.02 mol/l bis-Tris, pH 6. The transferrin was eluted with a salt gradient in the equilibration buffer from 0 to 0.1 mol/l NaCl in 10 min and 0.1 to 1 mol/l NaCl in 2 min at a flow rate of 1 ml/min.

The protein glycans were removed by enzymatic hydrolysis with N-glycosidase F followed by purification and desalting of the glycans by chromatography and gel filtration. The glycans were permethylated and their masses were determined by MALDI-TOF mass spectrometry (Ciucanu and Kerek, 1984). Electrospray mass spectrometry was used to study the mass of apotransferrin or of its glycoforms after separation by IEX-HPLC.

2 Serum iron parameters (II-V)

2.1 Patient serum samples

Patient serum samples were taken in clinical trials that were approved by the ethical committee of the Helsinki University Central Hospital and informed written consent of the patients was obtained.

2.2 Serum iron and transferrin saturation

Serum transferrin was determined by an immunoturbidimetric method. Serum iron was measured with colorimetric assays without deproteinisation using either ferene-S (Eskelinen *et al.*, 1983) or ferrozine (Eisenwiener *et al.*, 1979) as the chromogenic agents. The transferrin saturation was calculated by formula (3):

$$\frac{\text{Serum iron (µmol/l)}}{\text{Serum transferrin (g/l)}} \times 3.98 = \text{Transferrin saturation (\%)} \qquad (3)$$

Formula (3) is based on the maximal binding of 2 mol Fe^{3+} per 1 mol transferrin and a molecular weight of 79570 for transferrin (Morgan, 1992).

2.3 Determination of serum NTBI

The bleomycin-detectable iron assay by Evans and Halliwell (1994) was the primary assay for NTBI determination in publications II, III, and V, and it was adopted for a halved sample size. Additions were done in the following order: 250 µl 1 mg/ml DNA (Type I DNA from calf thymus, Sigma), 25 µl 1.5 IU/ml bleomycin sulphate (Sigma), 50 µl 50 mmol/l $MgCl_2$, 25 µl of sample, standard or blank and finally 50 µl 8 mmol/l ascorbic acid (Merck, Germany). A predetermined amount of about 7 µl 25 mmol/l HCl was added before the sample to adjust the pH of reagent mixture to 7.4. All reagents except the bleomycin were treated with Chelex (Bio-Rad) to remove excess iron. After incubation for 1 h at 37° C, 50 µl 0.1 mol/l EDTA, 250 µl 1% (w/v) thiobarbituric acid (Sigma), and 250 µl 25% (v/v) HCl were added and the mixture was incubated at 80°C for 20 min for chromogen formation. The pink chromogen complex was extracted with 1.5 ml butanol and the phases were separated by centrifugation. 350 µl of the clear top phase was transferred to microwell plates and the absorbance was measured at 535 nm using a microplate reader. 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 with 0.1 to 3 µmol/l

of iron (Iron atomic absorption standard solution in 1% HCl, Sigma Aldrich) was calculated for each series. Data was transformed to logarithmic values before calculation of the standard curve by linear regression in order to give weight to the standards with a low iron concentration (0.1-1 µmol/l) before fitting the curve.

The chelation method with NTA (Gosriwatana *et al.*, 1999) was used in publications II and IV. 100 µl 800 mmol/l NTA with pH 7 was added to 900 µl serum and the NTA-iron complex was recovered by ultrafiltration after 30 min at room temperature. The iron in the ultrafiltrate was quantified with a colorimetric assay with bathophenantroline chromogen or by atomic absorption spectrophotometry.

2.4 Determination of transferrin iron forms in serum

To study the transferrin iron forms in serum samples, the serum samples were either precipitated with rivanol before urea-PAGE, or the transferrin bands were visualised by immunoblotting after electrophoresis of serum. In both cases, the electrophoresis was carried out as described for the pure transferrin samples.

To obtain a transferrin fraction, 400 µl of 0.375% (w/v) rivanol (6,9-diamino-2-ethoxyacridine lactate, Sigma) with 10% (w/v) glycerol in TBE buffer was added to 50 µl of serum and the precipitate was removed by centrifugation (Williams and Moreton, 1980). Samples corresponding to 2 µl of serum were applied to the gel. Proteins were visualised with Coomassie Brilliant Blue staining.

For immunoblotting, serum samples with about 0.15 µg of transferrin were separated in 10x10 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 20% (v/v) methanol. The membrane was treated with 0.5% (v/v) Tween in PBS over night. Transferrin bands were visualised by immunostaining using rabbit anti-human transferrin IgG (Dako A/S) as the primary antibody in 1% (w/v) BSA, 0.05% (v/v) Tween 20 in PBS for 2 h at room temperature. The blots were washed three times with PBS containing 0.05% Tween 20 and incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Immuno Research Laboratories Inc) in 1% BSA, 0.05% Tween 20 in PBS for 1 h at room temperature. Following three additional washes with 0.05% Tween 20 in PBS, the blots were stained with 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium colour development solution (Immuno-Blot Alkaline Phosphatase Assay kit, Bio-Rad). The reaction was stopped with 100 mmol/l Na-acetate, pH 5, containing 5 mmol/l Na-EDTA for 5 min. The blots were washed with distilled water and dried.

The proportions of the different transferrin iron forms were determined by scanning with a laser densitometer.

3 Growth of *Staphylococcus epidermidis* (IV, V)

3.1 Bacterial strains and preparation of inoculum

Two multi-resistant clinical isolates of *S. epidermidis* (16779 and 19435) from neutropenic patients with a haematological malignancy and septicaemia and the ATCC 12228 strain (Manassas, VA, USA) were used. Stock cultures were kept frozen at -70°C and subcultures were made on trypcase-soy agar dishes (bioMérieux, France). The growth medium was RPMI-1640 (R-7509, Sigma) with 50 mmol/l HEPES buffer, pH 7.4 and 2 mmol/l L-glutamine. The medium was depleted of iron using Chelex-100 resin (Bio-Rad) and supplemented with 10 µmol/l $CaCl_2$ and 100 µmol/l $MgCl_2$. Ferric nitrilotriacetate (FeNTA) with 3 mmol/l iron was used for iron addition in precultivation of the strains and for saturating normal serum with iron.

To prepare the inoculum, the strains were cultivated in the iron-depleted RPMI with or without iron supplementation. The iron-depleted RPMI was used to induce the siderophore production. The siderophore index of the bacterial cultures was determined with the Chrome Azurol S liquid assay (Payne, 1994). 10-fold dilutions of the bacteria in saline were used as inocula.

3.2 Monitoring of growth with pure transferrin

Inocula with and without siderophore induction were used. 50 µl was used to inoculate 250 µl of RPMI containing 2.5 g/l of apotransferrin and different amounts of FeNTA. The growth was monitored by turbidity measurements every 30 min in microwell plates for 96 h at 37°C with periodic shaking in a Bioscreen C Microbiology Reader (Labsystems, Finland). Viable counts were determined by plating on trypcase-soy agar plates (bioMérieux, France).

3.3 Monitoring of growth in serum samples

The inoculum was cultivated in two passages, the first one in iron replete RPMI and the second one in iron-depleted RPMI. Serum was buffered with 50 mmol/l HEPES, pH 7.4. 50 µl inoculum was added to 200 µl buffered serum. The growth was monitored by optical density measurements every 30 min for 24 h at 37°C with periodic shaking in the Bioscreen C Microbiology Reader. The apparatus was placed in an air-tight chamber

filled with 5% CO_2 to retain the pH and bicarbonate levels of serum. For patient samples, the extent of growth was calculated from the change in OD during growth by subtracting the baseline OD from the peak level reached at the end of the growth, and was expressed as ΔOD. Viable counts were determined by plating on trypcase-soy agar plates (bioMérieux, France).

RESULTS

1 Pharmaceutical apotransferrin preparation (I)

1.1 Large scale purification of apotransferrin

In fraction IV, transferrin comprises 20-30% of the total protein and the main impurity is albumin (approx. 50%). The bulk of the albumin and other impurities were removed in the flow-through fraction of the first chromatography step, the cation exchange (Fig. 3). By using a pH-dependent elution in this chromatography, no buffer exchange was needed before the second chromatography, the anion exchange. This chromatography step was the final purification step. It was preceded by the solvent-detergent (SD) virus inactivation step and the SD chemicals were removed in this step by extensive column flushing before elution. In the eluate recovered from the anion exchange step, apotransferrin with a protein purity of at least 98% was obtained (Fig. 3). Later steps, including the diafiltration, concentration and the virus removal filtration, did not significantly increase the protein purity. In the overall process, over 90% of apotransferrin could be recovered from the dissolved fraction IV solution.

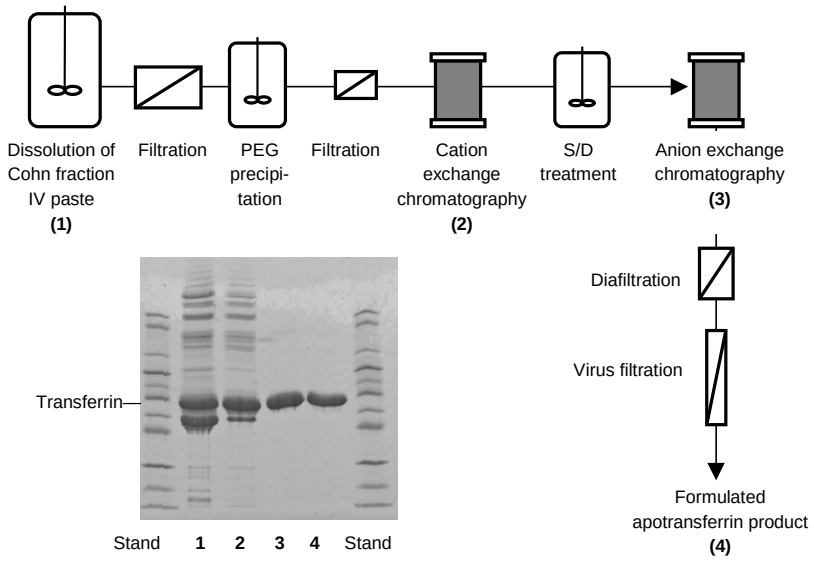

Figure 3. Flow scheme of the apotransferrin manufacturing process and SDS-PAGE purity analysis gel (non-reduced, Coomassie stained) with samples taken from the purification. Lane 1, dissolved fraction IV; Lane 2: partially purified apotransferrin after cation exchange; Lane 3: purified apotransferrin after anion exchange; Lane 4: formulated finished product.

By dissolving the fraction IV paste in water, the pH of the solution remained at the same level of pH 5.7 as used in the ethanol precipitation. Evidently due to the low pH and citrate buffer used for fraction IV precipitation, the iron was mostly dissociated from transferrin in the dissolved fraction IV. By adding EDTA to the solution and applying the solution to the first ion exchange at the low pH, iron was effectively removed by the wash fractions and apotransferrin with a low iron content corresponding to about 0.3% saturation could be recovered.

The specific virus inactivation and removal steps in the process comprised the SD treatment, the virus filtration and the PEG precipitation. Virus validation studies showed that the SD treatment rapidly inactivated the enveloped viruses HIV and BVDV with $\geq$4.7 $\log_{10}$ and $\geq$5.1 $\log_{10}$ reduction factors, respectively. The removal of the non-enveloped parvovirus B19 was studied in spiking experiments using PCR for virus detection. The results indicated that 3.7 $\log_{10}$ was removed in the PEG precipitation step and >4.5 $\log_{10}$ in the virus filtration. The virus filtration step could be carried out with high recovery at a high protein load of 4 kg/m^2.

1.2 Identification and characterisation of the apotransferrin product

The first 25 amino acids in the N-terminal sequence of the purified product showed identity with human transferrin. The identity was also confirmed with mass mapping with MALDI-TOF of the peptides after cleavage with endoproteinase-LysC, and revealed over 90% of the expected apotransferrin peptides. Electrospray mass spectrometry of the protein indicated a mass of the protein of 79556, which is the same as the calculated mass of 79555.4 of the apotransferrin molecule with two biantennary disialylated glycans. The mass was calculated from the polypeptide chain (75181.4 Da) with 19 disulphide bonds (-38 Da), two glycans (2x2224 Da) and glycan linkage (-36 Da). IEX-HPLC was used to separate the transferrin into four peaks. The IEX-HPLC gave reproducible results for different transferrin batches. The proportions of the main peaks (peaks 2-4) were 7-9%, 79-83% and 9-12% respectively (Fig. 4). The masses of the peaks and the glycan structures to which they resembled closely to are presented in Fig. 4.

For more detailed glycan analysis, glycan chains were enzymatically removed from apotransferrin and characterised by MALDI-TOF mass spectrometry. Glycan analysis confirmed the presence of the glycan structures in the IEX-HPLC peaks as predicted by electrospray mass spectrometry. Glycan analysis also revealed traces of structures carrying fucose in all IEX-HPLC peaks. Minor oxidation of single amino acids were found for some of the mass structures. The mass spectrometric studies revealed no

chemical modifications other than glycosylation and the presence of the predicted disulphide bonds, except minor oxidation.

The product purity and the protein integrity and conformation was studied for several production batches. The results indicated here are mean results of four batches. In SEC-HPLC the product was separated into a major peak of 98.5% corresponding to transferrin monomer and a minor peak, which corresponded to a molecular size of 180-290 kDa. The non-monomeric peak contained mainly IgA and IgG and a small amount of transferrin dimer. No polymers or fragments were found. The product showed a major band of 76 kDa in SDS-PAGE. The iron binding capacity was between 92% and 96% for different batches. The assay proved reproducible with imprecision CVs of 3% and 1%, inter- and intra-assay, respectively. The method was used in combination with urea-PAGE, which showed that the iron-free apotransferrin was fully converted into the iron-saturated form when iron was added. The iron content of the product was low, corresponding to approximately 0.3% transferrin saturation. No zinc or aluminium could be detected. The major plasma-derived impurities were hemopexin (0.6%), IgA (0.7%) or IgG (0.3%). Protease contaminants (plasmin/plasminogen and prekallikrein activator) were all below the detection limits of the assays.

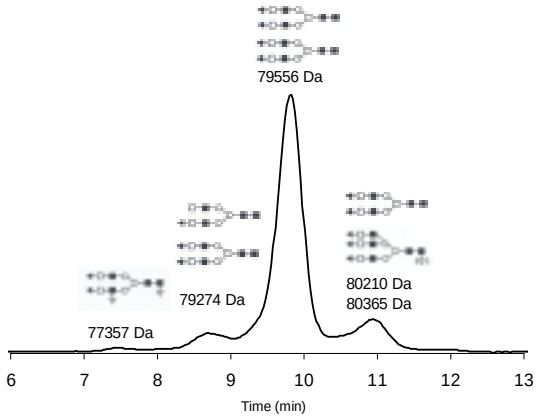

Figure 4. Apotransferrin separated into four glycoform peaks by IEX-HPLC. The molecular weight of apotransferrin in the peaks was measured by electrospray mass spectrometry and the proposed glycan chains are shown. The symbols for the monosaccharide residues are: (◄) N-acetylneuraminic acid; (□) galactose; (■) N-acetylglucosamine; (○) mannose and (∇) fucose.

The stability of the 50 g/l liquid formulation was studied. The most informative assays to detect changes in the product proved to be the iron binding capacity, the transferrin iron forms by urea-PAGE, IEX-HPLC and SEC-HPLC. The liquid product proved to be stable for up to 2 years at refrigerator (2-8°C) and 12 months at room temperature. Lyophilised formulations were stable for four years at refrigerator temperature. The finished product was sterile, had a low endotoxin content (<2 IU/ml) and was pyrogen free according to the European Pharmacopoeia methods.

2 Binding of NTBI with apotransferrin

2.1 Evaluation of assay for measuring NTBI (II)

In the bleomycin assay, the sample and reagent volumes were halved and absorbance measurements were done in microwell plates. The bleomycin assay proved reproducible. The imprecision coefficients of variation (CVs) were 7.7% and 8.2% intraassay and 18.4% and 9.8% interassay for a low (0.2 µmol/l) and a high (1.5 µmol/l) control, respectively. To study the assay accuracy, ferric nitrilotriacetate (FeNTA) was added to saturate transferrin in serum and the full saturation point was confirmed by urea-PAGE. The NTBI was ≥0.1 µmol/l in samples with full saturation. The recovery of the added iron was only ~33% in samples with full transferrin saturation. The interference of sample haemolysis was studied. The haemolysis had a clear raising effect on NTBI, but had no effect on the transferrin saturation or on the total iron. The degree of haemolysis was studied visually and by haemoglobin determination. In samples with non-visible haemolysis (haemoglobin 290-645 mg/l) up to 0.07 µmol/l NTBI could be measured. No interference of ferritin was found when patient samples containing high ferritin concentrations were studied, nor was the treatment drug cyclophosphamide found to interfere with the assay. A detection limit of 0.1 µmol/l could be established based on accuracy studies and on the interference of non-visible haemolysis.

399 patient serum samples were studied and the NTBI levels were compared with transferrin saturation values. NTBI (≥0.1 µmol/l) was found only when transferrin saturation was >80%. When samples with visible haemolysis were excluded, there were no positive samples with <80% transferrin saturation. The NTBI varied between 0 and 0.97 µmol/l in patient samples without visible haemolysis. The highest NTBI concentration measured with the bleomycin assay in a haemolysed patient sample was 1.6 µmol/l.

The bleomycin assay was compared with the chelation method for measuring NTBI. The detection limit of the chelation assay was 1.5 µmol/l and the recovery of iron in

serum was about 64%. The chelation method gave clearly higher NTBI concentrations (up to 10-14 µmol/l) than the bleomycin assay (up to 1.27 µmol/l) in 61 samples studied in parallel. There was no clear correlation between the quantitative results of the two assays in samples above the detection limit of the respective assays. Due to a low sensitivity (0.01 AU/µmol/l) of the colorimetric determination of the chelated iron, iron quantification in the ultrafiltrate was also studied by AAS. The AAS gave similar results as the colorimetric assay, and verified the higher NTBI concentration compared with the bleomycin assay. However, the chelation method gave positive results for some samples also at <80% transferrin saturation. In these samples the partial transferrin saturation was confirmed by urea-PAGE.

2.2 Binding of NTBI in patient serum (III, V)

Apotransferrin was administered to SCT patients in clinical trials. In the first trial (III), a single dose of 100 mg/kg was given to six patients three days after the SCT. Serum samples taken before and after the administration and the serum iron parameters including NTBI and urea-PAGE were studied. Initially, all patients had serum transferrin saturation >80% and NTBI in their serum. 15 min after the apotransferrin injection, serum NTBI had disappeared in all patients and transferrin saturation decreased to 30-50%. The mean increase in the serum transferrin concentration was 1.95 g/l.

Before apotransferrin administration, only fully saturated diferric transferrin was found in serum, shown by urea-PAGE. 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. 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 different rates in the individual patients and coincided with the increase in the calculated transferrin saturation. The amount of iron bound by the administered apotransferrin during the first 15 min was on the average 16 µmol/l (range 11-22 µmol/l), when calculated from the amount of formed monoferric transferrin. This was much higher than the NTBI concentrations measured by the bleomycin-assay before the administration (0.3 ± 0.1µmol/l, mean ± SD) (II).

NTBI reappeared and the transferrin saturation exceeded 80% 12-48 h after the injection in four patients and after six days in one patient. NTBI remained non-detectable for the whole 12-day follow-up period in one patient.

Apotransferrin was also given in repeated doses to twenty patients, starting six days before the SCT, before the onset of myeloablative conditioning which leads to appearance of NTBI in patient sera. Three different dosage regimens were studied during a three-week follow-up period, the total doses per patient were 0.3, 0.6 and 1 g/kg, respectively. With the repeated doses, the occurrence of NTBI could be completely avoided in 5 of 8 patients who received 1 g/kg of apotransferrin and in one patient who received 0.3 g/kg. In the other patients, the apotransferrin dosing was not sufficient to completely prevent full transferrin saturation and NTBI appearance during the follow-up period.

3 Growth of *Staphylococcus epidermidis* (IV, V)

The growth of *S. epidermidis* in serum-free conditions in the presence of pure transferrin at different saturation levels was studied. The transferrin saturation was monitored by absorbance measurements and the transferrin iron forms by urea-PAGE. At low initial bacterial cell densities (10^2 - 10^4 CFU/ml), the growth of *S. epidermidis* was critically dependent on NTBI and a high transferrin saturation of at least 90%. At higher initial densities, growth was detected also in the presence of partially saturated transferrin. The siderophore induction measured as the siderophore index was highest when the inoculum was precultivated in iron-depleted media. Addition of FeNTA up to 1 µmol/l resulted in a linear decrease of the siderophore index. Induction of siderophore production during precultivation did not change the growth dependence on NTBI. All three *S. epidermidis* strains showed the same dependence on full transferrin saturation for growth.

In serum, a similar influence of NTBI on growth was detected. In normal serum samples with iron added to form NTBI and in patient samples with NTBI, *S. epidermidis* grew consistently at all inoculated (10^2 – 10^5 CFU/ml) cell concentrations. In normal serum, no growth was detected at inoculated cell densities up to 10^3 CFU/ml. At higher initial densities, growth was detected but with a slower rate than in the samples with NTBI. Addition of apotransferrin to bind NTBI restored the growth inhibitory effect, whereas addition of holotransferrin did not. Again, the three *S. epidermidis* strains behaved similarly.

The ability of *S. epidermidis* to grow in samples from SCT patients was studied. Using one of the multiresistant strains, bacteria (10^3 CFU/ml) were inoculated to serum

samples and the growth was measured as the increase in OD from baseline to its maximal level, expressed as ΔOD. The sensitivity of the OD assay to detect growth in serum samples was verified by viable count measurements. The detection limit for significant growth corresponded to a ΔOD value of 0.05, which was calculated as 10 SD above the background ΔOD value of serum samples (*n*=18) without growth curve. The threshold density of *S. epidermidis* which could be detected by the OD determination was about 10^6 CFU/ml and thus a positive ΔOD value indicated growth of at least 3 $\log_{10}$ in 24 h.

The bacterial growth studied in 132 patient samples was found to have a close relation to the presence of NTBI and a high transferrin saturation level (>80%). In samples from two patients who received no apotransferrin, the bacterial growth was promoted at the same time as NTBI appeared in serum due to myeolobalative treatment. In three patients who received a single dose of apotransferrin the growth was prevented simultaneously as the NTBI was bound by transferrin. The bacteriostatic effect typically disappeared when NTBI reappeared. A patient who received repeated apotransferrin in high enough doses to prevent formation of NTBI also had restored bacteriostatic effect in serum throughout the follow-up period.

Markers for septic infections (fever >38°C, clearly elevated CRP >30 mg/l) were compared in the patients who did not receive apotransferrin or received repeated doses of apotransferrin at two different dose levels, 0.3-0.6 g/kg and 1.0 g/kg respectively. There was a trend, however not statistically significant, towards a lower incidence of suspected septic infections among the patients who received high apotransferrin doses compared with the patients who did not receive apotransferrin. The apotransferrin administrations reduced in a dose-dependent manner the number of days with detectable NTBI in the patient serum. When the total number of days with positive infection markers was evaluated as a proportion of all study days, there was a significant reduction in the number of days with fever and days with elevated CRP in the patient groups who received low and high doses of apotransferrin, respectively. When both markers were evaluated together, there was a significant reduction in the high dose group.

DISCUSSION

1 Apotransferrin product (I)

The developed manufacturing process enabled production of pure and virus-safe apotransferrin with high yield and only a few process steps. Transferrin was maintained in the iron-free apoform from the beginning of the process, and excess iron was removed in the first ion exchange step. It was therefore not necessary to have a separate iron-removal step. Apotransferrin was purified by a combination of a cation and a anion exchange chromatography. Only one ultrafiltration step was needed for concentration and buffer exchange of the pure apotransferrin solution.

The viral safety of the apotransferrin product was based on reduction of potential virus load in the starting material by donor selection and testing for infection markers and by effective virus inactivation and removal during the manufacturing. The major virus inactivation step in the process was SD treatment, which very reliably inactivates all enveloped viruses (Horowitz *et al.*, 1993; Pehta, 1996). Virus filtration with a 15 nm pore size hollow fibre filter effectively removes even the small non-enveloped viruses, such as HAV and parvovirus (Burnouf-Radosevich *et al.*, 1994). Parvovirus B19 currently remains a problem with plasma products because its level in the starting plasma may be very high (up to10^9 gen. eq./ml) and a single virus filtration step may not be capable of removing the potential virus load from the plasma pools (Siegl and Cassinotti, 1998). Although clinically significant parvovirus transmissions by plasma products are apparently very rare, parvovirus transmission might be harmful to immuno-compromised patients receiving high-dose chemotherapy (Schmidt *et al.*, 2001), who participated in this study. Therefore, the starting plasma was tested for parvovirus B 19 by PCR as a complementary measure on the overall virus safety. Both PEG precipitation and 15 nm virus filtration were implemented to the process in order to achieve two effective removal steps for parvovirus. Considering the potential level of parvovirus in the plasma pools after PCR screening and the clearance of parvovirus observed in virus removal studies of the process, it could be concluded that the finished product was with high confidence safe also with respect to parvovirus transmission.

The structural characterisation indicated that the pure apotransferrin product was identical with human plasma iron-free transferrin. Electrospray mass spectrometry did not indicate chemical modifications in the apotransferrin molecule other than the N-linked glycans and the predicted 19 disulphide bonds, except minor oxidation most probably of single methionine residues. The identified glycan chains were structurally closely similar to the structures described before for human plasma transferrin (Fu and

van Halbeek, 1992). The minor transferrin variant with only one biantennary glycan chain has not been identified in the earlier studies.

The process yielded a pharmaceutical composition of apotransferrin, which had a purity of at least 98%, contained mainly monomeric transferrin and had no detectable polymers or aggregates. Assays were developed for the assessment of the iron binding capacity and integrity of the pure apotransferrin product, which proved to be sensitive indicators of the stability of the product. Both the lyophilised formulations and the liquid formulation proved to have good stability at refrigerator temperature, four and two years respectively. The developed transferrin assays were also used to study the quality of the finished product. Results of products from several batches confirmed that the production method could be carried out with consistent results for the finished product, which is a prerequisite for a production method of a medicinal product. The formulation with sodium chloride at pH 6 was suitable for intravenous administration and the product sterility and process hygiene controls were confirmed by the pharmacopoeia methods.

Potentially harmful impurities described in other transferrin products include aggregates and polymers formed during pasteurisation (Haupt, 1993). Presence of high amounts of process derived aluminium (Rigal *et al.*, 1989) and zinc (Rolf *et al.*, 1997) has been reported. The product with a high aluminium content was used in a study with three patients undergoing myeloablative chemotherapy and bone marrow transplantation. Two of the three patients developed acute renal failure, which may have been associated with the administration of the high aluminium content of the transferrin preparation. The study did not demonstrate efficacy of the transferrin preparation in the binding of free iron in the patients or other benefits, but rather suggested that the therapeutic use of transferrin may not be safe. As with other plasma derivatives, contaminating plasma proteases present a further possible cause for adverse effects in patients. However, the level of all these plasma and process derived impurities was very low in the present apotransferrin product.

2 Determination of serum NTBI (II, III)

The bleomycin assay evaluation demonstrated that the microwell modification of the assay could be carried out reproducibly using one-half the amount of reagents and serum compared with the original assay (Evans and Halliwell, 1994), which made it possible to study large numbers of patient samples in microwell plates. Sample haemolysis interfered with the assay, and there was a clear correlation between the NTBI and the haemoglobin concentrations. An earlier report indicated that pure haemoglobin does not interfere with the assay (Gutteridge *et al.*, 1981). It is possible,

that with the mechanical haemolysis studied here other degradation products from haemoglobin were formed which could have caused the interference. However, the non-visible haemolysis did not result in false positives when using a detection limit of 0.1 µmol/l, which was also the detection limit which was measured in the accuracy studies.

When the bleomycin assay was compared with the chelation method for measuring NTBI, it was found that the latter one gave NTBI concentrations that were clearly higher (max 14 µmol/l) than with the bleomycin assay (max 1.6 µmol/l). Similar NTBI concentrations in patient samples obtained by the bleomycin assay have been found by (Carmine *et al.*, 1995), whereas levels of 20-28 µmol/l have been measured by others (Halliwell *et al.*, 1988; Harrison *et al.*, 1994). The higher total levels were found with an earlier version of the bleomycin assay (Gutteridge *et al.*, 1981) without the final butanol extraction step, which apparently reduces the risk of possible interfering compounds in the spectrophotometric measurement. Using the chelation method the NTBI concentrations were in a similar range of 0-10 µmol/l as found by others using the same type of assay (Bradley *et al.*, 1997; Gosriwatana *et al.*, 1999).

It was also found that unlike the bleomycin assay in which positive samples were detected only when transferrin saturation was >80%, the chelation method detected NTBI in some serum samples also when the transferrin saturation was <80%. The use of a chelator to mobilize NTBI in serum samples evidently is a reason for the higher NTBI levels gained by the chelation-based assay. The chelator probably mobilises iron from complexes in which it is not available for the bleomycin reagent. 80 mmol/l NTA was able to mobilise iron from albumin, but also in small amounts from other proteins such as transferrin (~0.5 µmol/l) and ferritin (~0.3 µmol/l) (Gosriwatana *et al.*, 1999). The use of the chelating agent could also explain our results of detectable NTBI in serum samples with <80% transferrin saturation, if the chelating agent had mobilised a small amount of transferrin-bound iron.

In the patients who received a single apotransferrin dose, the level of NTBI was also calculated from the amount of iron bound to the administered apotransferrin, based on the shift of apotransferrin into the monoferric form measured by urea-PAGE 15 min after the injection. 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 found by the chelation method. It is possible that the intravenously administered apotransferrin had the capacity to mobilise NTBI from complexes in the vessel wall. Such loosely bound iron cannot be measured in serum samples. The vascular walls can theoretically provide polyanionic sites for NTBI, such as glycosaminoglycan chains of endothelial surface proteoglycans. Another speculation for the high concentration of NTBI calculated in the 15 min samples, is that the apotransferrin was able to immediately sequester low-molecular-weight iron which is released by macrophages

from degrading erythrocytes (Fillet *et al.*, 1989; Moura *et al.*, 1998). Instead of being at least partly taken up by the transferrin independent uptake mechanism in parenchymal and especially liver cells (Craven *et al.*, 1987; Kaplan, 2002), the iron could be directly bound by the apotransferrin which was available after the infusion.

The results of the different methods to measure NTBI indicate that the bleomycin assay does not measure the total amount of NTBI. The true concentration of NTBI in serum of the SCT patients can most likely be in the order of 10 µmol/l, even up to 20 µmol/l, as measured by the chelation method and calculated as the iron bound to injected apotransferrin *in vivo*. However, the bleomycin assay has high specificity and does not measure ferritin iron, which makes it a useful method to detect NTBI. The NTBI measured by the bleomycin assay is capable of binding to bleomycin and generating free radicals and is therefore considered to be a measure of the redox activity of NTBI. The lower concentrations obtained with the bleomycin assay reflects the heterogeneous nature of NTBI being a mixture of different iron forms with different affinities for different chelators (Hider, 2002; Petrat *et al.*, 2002). It could mean that only a portion of the loosely bound NTBI measured in serum is redox-active and capable of binding to bleomycin whereas a larger portion can be chelated by NTA.

3 Binding of NTBI by apotransferrin administration (III, V)

In the patients who received single dose injections of apotransferrin, NTBI measured by the bleomycin assay disappeared from the sera of all patients indicating that it was effectively bound by the administered apotransferrin. NTBI remained undetectable 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. 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. Complete prevention of NTBI during the whole studied SCT period could be achieved with repeated administrations starting before the onset of the myeloablative treatment.

Apparently 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 thalassemia major. It has been shown that part 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). However, our results with the bleomycin assay did not indicate the presence of NTBI unless transferrin saturation exceeded 80%. 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. It is possible that the underlying disease is the reason for the differences in NTBI forms found in the different patients groups. In haemochromatosis, the NTBI is formed after a slow accumulation of body iron stores over a long time period of several, even decades of years. In the haematological patients studied in this work, a halt of erythropoiesis and release of iron from senescent erythrocytes form the NTBI within hours. It may be, that the gradual formation and long presence of NTBI in the haemochromatitic patients may lead to less soluble and perhaps oligomeric iron isoforms that have a low affinity for transferrin or desferrioxamine (Hider, 2002).

The results indicate the feasibility of using apotransferrin as a natural iron binding agent for the SCT patients. In contrast to chelation by desferrioxamine, whereby iron is excreted from the body (Porter, 2001), the recovering erythropoetic cells of the bone marrow could utilise transferrin-iron after the stem cell transplantation. The clinical efficacy of the apotransferrin was not within the scope of this work and remains to be studied in larger patient groups. The results from the biochemical efficacy studied in this work indicate that doses of apotransferrin of 1 g/kg, or even higher, will be needed to prevent NTBI formation in the SCT patients.

4 Transferrin, NTBI and the growth of *Staphylococcus epidermidis* (IV,V)

In serum free conditions with transferrin, the growth of *S. epidermidis* was critically dependent on NTBI and high transferrin saturation for growth. A similar effect was found when the growth in serum was studied. At inoculated cell densities of 10^4 CFU/ml and higher, growth could be detected but with a slower growth rate than in serum samples that had full transferrin saturation and NTBI. When the growth in SCT patient samples was studied, 80% was found to be a threshold for transferrin saturation, above which bacteria grew. There was also a strong association between bacterial growth and NTBI.

Earlier studies of growth of *S. epidermidis* in serum free conditions have indicated growth in the presence of iron-saturated transferrin (Lindsay *et al.*, 1995; Modun *et al.*, 1998), which is in line with the present studies. It was suggested that a cell wall receptor identified as glyceraldehyd-3-phosphate dehydrogenase (GAPDH) acts as a transferrin receptor and facilitates iron removal from the receptor-bound transferrin (Modun *et al.*, 2000). In these studies, high (10^8 CFU/ml) bacterial densities were used. In our studies, the inability of the bacteria to grow at low initial densities in the presence of partially saturated transferrin suggests that iron displacement from transferrin requires high bacterial concentrations. It is thus possible, that the receptor mediated mechanism to

scavenge iron from transferrin requires presence of high cell densities, perhaps thereby forming high concentrations of organic phosphates that might displace iron from transferrin (Morgan, 1979). The GAPDH receptor-mediated facilitation of iron was recently questioned in the case of *S. aureus* by Taylor and Heinrichs (2002). They found that GAPDH showed no affinity for transferrin, and a new cell wall protein was described as the transferrin receptor. Unlike the GAPDH, this protein was expressed only in iron-limiting conditions. The role of GAPDH as a receptor for transferrin in the staphylococci is therefore probably not fully clarified. It is possible that the role of GAPDH in iron transport is mainly due to its capability to form the phosphate compounds that at high concentrations can release iron from transferrin.

The growth of *S. epidermidis* was studied in samples taken from SCT patients who received different doses of apotransferrin or no administration. The appearance of NTBI and loss of growth inhibition coincided when analysed in sequential serum samples taken after initiation of myeloablative conditioning. Intravenously administered apotransferrin that effectively bound NTBI also restored the growth inhibition. Repeated administrations of apotransferrin could in high doses maintain the growth inhibitory effect during the whole stem cell transplantation period.

Because the ability of *S. epidermidis* to multiply in serum in the absence of NTBI was dependent on the initial bacterial density, the initial bacterial count may play a role in the pathogenesis of septic infections. *S. epidermidis* often contaminates central venous catheters, where biofilm formation promotes the adherence and protection of the bacteria (Kloos and Bannerman, 1994). In stem cell transplant patients, the oral mucosa is another possible route of infection due to treatment-related mucositis (Kennedy *et al.*, 2000). In catheter infections, there is an association between the number of organisms retrieved from the catheter surface and the risk for infection, and infection occurs only when the number exceeds a certain quantitative threshold (Raad, 1998; Sherertz *et al.*, 1990). In a study of catheter-related bacteremias, blood drawn from the catheters had up to 10^5 CFU/ml of coagulase-negative staphylococci, whereas peripheral blood samples had 10^1 – 10^2 CFU/ml (Flynn *et al.*, 1987). Another study showed that the mean bacterial cell concentration in peripheral blood samples in blood stream infections caused by coagulase-negative staphylococci was 33 CFU/ml (Herwaldt *et al.*, 1996). It may be reasoned, that the high local density of *S. epidermidis* in the intravenous catheters promotes bacterial growth also in the absence of NTBI, whereas the proliferation of the low counts occurring in the peripheral blood may depend on the availability of NTBI.

Among the small number of patients in the present study, the incidence of septic infections was not significantly different in the patients who received apotransferrin than in the control patients. When the patient groups were compared with respect to the

total number of days with fever or elevated CRP, the patients who received repeated doses of apotransferrin had significantly less positive days than the patients who did not receive apotransferrin. Correspondingly, the number of days with intravenous antibiotics was significantly smaller during the study period in the patients who received repeated apotransferrin infusions. Although the patients were not compared in a randomised setting, this suggests that repeated infusions of apotransferrin may have protected the neutropenic patients against septic infections.

CONCLUSIONS

A manufacturing method was developed whereby human apotransferrin can be produced with high recovery from Cohn fraction IV with only a few process steps. The product has high purity and a native conformation and is suitable for intravenous infusion. A stable liquid formulation was developed. The production method comprises virus inactivation and removal steps, which with high confidence contribute to the virus safety of the product.

The microwell modification of the bleomycin assay for NTBI determination is reproducible and has a high specificity to detect NTBI in serum samples at >80% transferrin saturation, when haemolysed samples are excluded. The bleomycin assay measures redox-active iron, but it underestimates the true concentration of NTBI. The concentration of NTBI can be calculated from the shift of transferrin iron forms found *in vivo* after intravenous infusion of apotransferrin to patients. It can also be determined with the chelation based method, which, however, has a lower specificity than the bleomycin method. In the SCT patients, the concentration of NTBI can be as high as 20 µmol/l.

Apotransferrin given in single intravenous doses to six patients bound NTBI effectively, although in most cases temporarily. With repeated high dose regimens, the appearance of NTBI could be prevented in 5 of 8 patients.

The opportunistic pathogen *S. epidermidis* is dependent on NTBI for growth at low bacterial concentrations. Apotransferrin administered to patients binds NTBI and restores the growth inhibitory effect of serum. Apotransferrin might protect the patients against infections by *S. epidermidis* and other opportunistic pathogens whose growth is dependent on NTBI.

In conclusion, the apotransferrin is pure and safe and shows *in vivo* the biochemical effects that can be expected of a functional human apotransferrin product. In SCT patients it is possible to prevent the appearance of NTBI and maintain the bacterial growth inhibitory effect in serum.

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