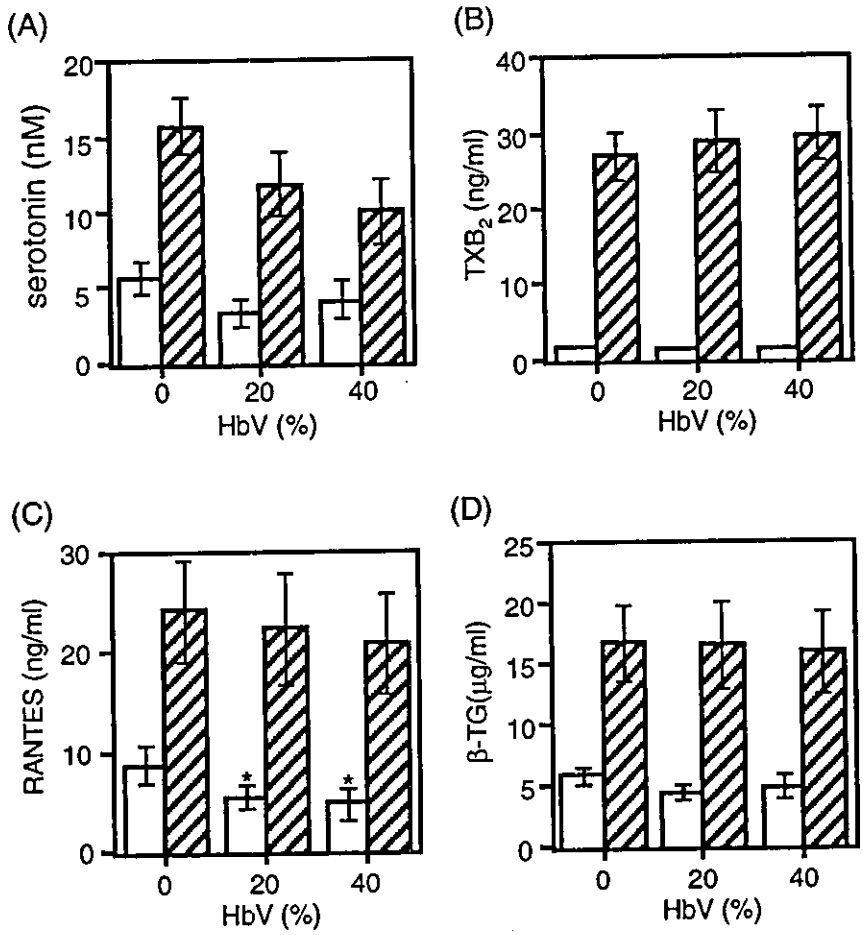

Fig. 3

Wakamoto et al.

総　説

血液事業の新しい動き
—8項目の安全強化対策について—

New Movement of Blood Program
—Eight Strategies for the Sake of Safer Blood Products—

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和文抄録

2005年4月に完全施行される改正薬事法では，ヒトその他の生物に由来するものを原材料として製造される医薬品および医療機器等のうち保健衛生上，特別の注意を要するものを生物由来製品と定義し，さらにその中に特定生物由来製品というものを定義した．これは生物由来製品のうち市販後に当該製品による保健衛生上の危害の発生または拡大を防止するための措置を講ずることが必要なものを指す．全ての血液製剤を含めて50品目がこの特定生物由来製品に入る．日本で検討されているリポソーム包埋型の人工赤血球が，ヒト由来のヘモグロビンを使用している以上は，この特定生物由来製品の範疇に入る可能性があると考えられる．従って現在，日赤で取り組もうとしている安全強化対策がどのようなものであるかを知ることは重要であろう．そこで，本稿では日本赤十字社が，昨年から，取り組むことを宣言している8項目の血液製剤の安全性強化対策1）遡及調査自主ガイドラインの作成，2）新鮮凍結血漿の貯留保管の実施，3）輸血用血液の感染性因子の不活化　4）核酸増幅検査の精度向上，5）複数地域の医療機関の協力を得ての輸血後感染症に関する全数調査の実施，6）E型肝炎ウイルスの疫学的調査の実施，7）保存前白血球除去の開始，8）献血受け付け時の本人確認の実施への試行，について，その経緯も振り返りながら解説する．

Keywords

Look back, Quarantine, Inactivation, NAT, Hepatitis E virus, Prestorage leukoreduction

Abstract

In the revised Pharmaceutical Affairs Law that is supposed to become completely effective as from April 2005, it is described that among pharmaceuticals and medical equipments made from materials derived from human and other animals, those to which we should pay special attention with regards to health and hygiene are defined as biological products. In addition, among biological products, the specific biological products are defined. Once the specific biological products are on the market, one should take actions to prevent the occurrence and/or spread of any damages to health and hygiene caused by these products. Fifty items including all the blood products are recognized as specific biological-products. Since liposome encapsulated artificial red blood cells (ARCs) in Japan contains hemoglobin molecules which are derived from human red blood cells, it is probable that this type of ARCs is also categorized as a specific biological product. Therefore, it may be important for us to share the information on the eight strategies that Japanese Red Cross is about to take to enhance the safety of blood products.

始めに

日本赤十字社は2004年10月1日，輸血用血液へのウイルス混入などがあったときに素早く意思決定して安全対策を行えるよう，社内で独立した権限を持つ「血液事業本部」を発足させ，その意思決定機関として「血液事業経営会議」を設置，この経営会議が血液事業全般の責任を持つこととした．今後は経営会議で血液の安全対策や効率的な経営などを話し合って方針を決める．

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血液事業の有り様は，ここ数年の間に大きく変化した．最大の変化は，血液新法と改正薬事法が施行されることである．まず，血液新法であるが，これは1956年（昭和31年）に制定された「採血および供血あっせん業取締法」が47年ぶりに改正され，「安全な血液製剤の安定供給の確保等に関する法律」として改訂されたものである．基本理念として血液製剤の安全性の向上，安定供給の確保，適正使用の推進が掲げられ，血液事業に携わる関係者，特に国の責任が明確化された．すなわち，国は，安全性の向上，安定供給の確保に関する基本的・総合的施策の策定・実施を行うことがうたわれている．ところで2003年７月にこの法律が施行される少し前に，輸血用血液製剤による感染症の発生と日赤の遡及調査の不備が厚生省により指摘されたことは偶然ではない．古い法律では，採血業は大臣の許可のもとに営むことができたのであり，法律上は，国は許可をする立場であり，現場の責任者として行動する必要性が無かったのである．法律の公布が確定することをきっかけとして，国は初めて，日赤が行ってきた血液のスクリーニングシステムや輸血感染症の実態に対して責任ある立場にたったということである．官として法律に従って動いたとも言える．

事の発端はともかく，血液製剤の安全性が今まで以上に厳しく問われ，膨大な数の遡及調査が行われ，日本に於ける輸血感染症の実態が把握されつつある．また，完璧な感染症スクリーニングというものも，現状ではあり得ないことが改めて関係者の知るところとなった訳である．

改正薬事法の完全施行は2005年４月からである．あらたな薬事法では，ヒトその他の生物に由来するものを原材料として製造される医薬品医療機器等のうち保健衛生上，特別の注意を要するもの（遺伝子組み換え製剤，ワクチン，トキソイドなど）を生物由来製品と定義し，さらにその中に特定生物由来製品というものを定義した．これは生物由来製品のうち市販後に当該製品による保健衛生上の危害の発生または拡大を防止するための措置を講ずることが必要なものを指す．具体的には全ての血液製剤，そして遺伝子組み換えの第８因子製剤あるいは第９因子製剤といった50品目がこの特定生物由来製品に入る．現在，日本で検討されているリポソーム包埋型の人工赤血球が，ヒト由来のヘモグロビンを使用している以上は，この特定生物由来製品の範疇に入る可能性が非常に高いと考えられる．すなわち通常の製剤としての安全性ではなく，特定生物由来製剤として，既存の血液製剤と同等かそれ以上の安全性を確保していることがどうしても期待されることになる．人工赤血球に使用するヘモグロビンのソースを何処に求めるかによっても，安全性対策の方向性が異なるわけであるが，少なくとも現時点では，ヒト由来のヘモグロビンを使うことになるので，現在，日赤で取り組もうとしている安全強化対策がどのようなものであるのかを知ることは重要であろう．

日本赤十字社では，昨年から，８項目の血液製剤の安全性強化対策に取り組むことを宣言している．その内容は

１）遡及調査自主ガイドラインの作成，
２）新鮮凍結血漿の貯留保管の実施，
３）輸血用血液の感染性因子の不活化
４）核酸増幅検査の精度向上，
５）複数地域の医療機関の協力を得ての輸血後感染症に関する全数調査の実施，
６）Ｅ型肝炎ウイルスの疫学的調査の実施，
７）保存前白血球除去の開始，
８）献血受け付け時の本人確認の実施への試行

である．以下に各項目について若干の解説を試みる．

[1] 遡及調査自主ガイドラインの作成

前述の如く，昨年厚生労働省より日赤の遡及調査（Look back）体制の不備を指摘され遡及調査自主ガイドラインを作成することになり，今年（2004年）の８月15日以降は，このガイドラインに基づいて遡及が行われている．その目的は，感染リスクがあると判断された血液製剤について１）それが未使用で有効期限内にあれば直ちに使用停止・回収を行ない，それによる感染の拡大を防ぐこと，２）既に輸血に使用されていた場合には，医療機関にその情報を提供し，その血液が輸血された患者の感染の有無を調査し，感染事実の早期発見，早期治療を可能にしかつ２次感染の防止を図ることである．それでは遡及の開始はどのような情報にもとづいているのかというと，１）献血者の感染症検査（血清学的検査または核酸増幅検査）が陽転化したという情報が得られた場合，２）献血後に献血者が感染症（肝炎等）を発症したという情報が得られた場合（本人からの自己申告等），３）医療機関からの（輸血後感染の）副作用報告の３つの情報に基づく．今回の遡及対象はB型肝炎ウイルス（Hepatitis B virus（HBV)），C型肝炎ウイルス（Hepatitis C virus（HCV)），ヒト免疫不全ウイルス（Human immunodeficiency virus（HIV)）そして梅毒トレポネーマの４種類であるが，その他の病原体に関してもこのガイドラインに準ずることとなった．以下にB型肝炎について説明するが，まずはじめに感染症マーカーの意味について解説する．従来の考え方では，急性B型肝炎を発症した人の多くは治癒する．完全に治癒した人は,ウイルス抗原であるHBsAg陰性かつ中和抗体であるHBs抗体が陽性で，血中のHBc抗体を測定すると値は低いが検出できるという状態であり，HBc抗体は，昔B型肝炎にかかったことを示す（既往の有無を示す）指標と考えられていた．これらはいずれもウイルスそのものを検出するのではなくあくまでも血清学的検査である．そしてHBc抗体価がある基準値以下の人の血液は，他の血清学的マーカーに問題がなければ輸血用血液として採用されている．一方，核酸増幅検査（Nucleic Acid Amplification Test（NAT））は血中のウイルスゲノム自体を高感度に検出できる検査法であり，血清学的検査法と比較して明らかに感度が高く，血清学的検査結果がまだ陰性である感染早期（ウイルス血症の時期に）陽性となり，血清学的検査が陽転化して治癒するとウイルスが消失するので陰性になる．一方抗体は治癒後も長く血中に存在し続ける．ウイルスに感染してから，血清学的検査あるいはNAT検査のそれぞれの検査が陽性となるまでの期間は，空白期間（Window period）と呼ばれている．血清学的検査の空白期間はNAT検

査の空白期間より長い．現在日赤ではすべての献血血液について従来の血清学的検査を行い，かつ血清学的検査に合格したものについてHBV,HCV, HIVのプールNAT検査を施行している．ところで，HBc抗体陽性を示す既感染者（治癒したと判断される）の中に，NAT検査をすると微量ながら血中にウイルスの存在している人のいることが最近わかってきている．非常に微量であるため，NAT検査でも結果が一定しないこともある．HBV感染の場合，まず，血清学的検査の空白期間は，検査法の感度にもよるが，現行の日赤スクリーニング法では125日，50プールNATの空白期間は理論的空白期間46日であるが，これは平均値なので，それを2倍した92日と定められた（表1）．50プールNATが陽転化しかつHBc抗体が高感度法（EIA等）でも検出されない，すなわち急性感染と判断された場合の遡及は最大で血清学的空白期間である125日過去まで遡ることとされた（図1）．なぜなら，この場合の感染時期は血清学的検査の空白期間内に必ず存在すると考えられるからである．その範囲内で前回（プールNAT陰性）およびそこから92日以内のすべての血液を遡及することとなった．一方，HBc抗体が高感度法で検出された場合は，既感染であるので可能な限り遡る．しかし，この場合は過去の感染時期を特定出来ないし，NAT検査で血中のウイルスが検出できたり出来なかったりする可能性がある．そこで保管検体の個別NATが陰性と判定されるまで遡

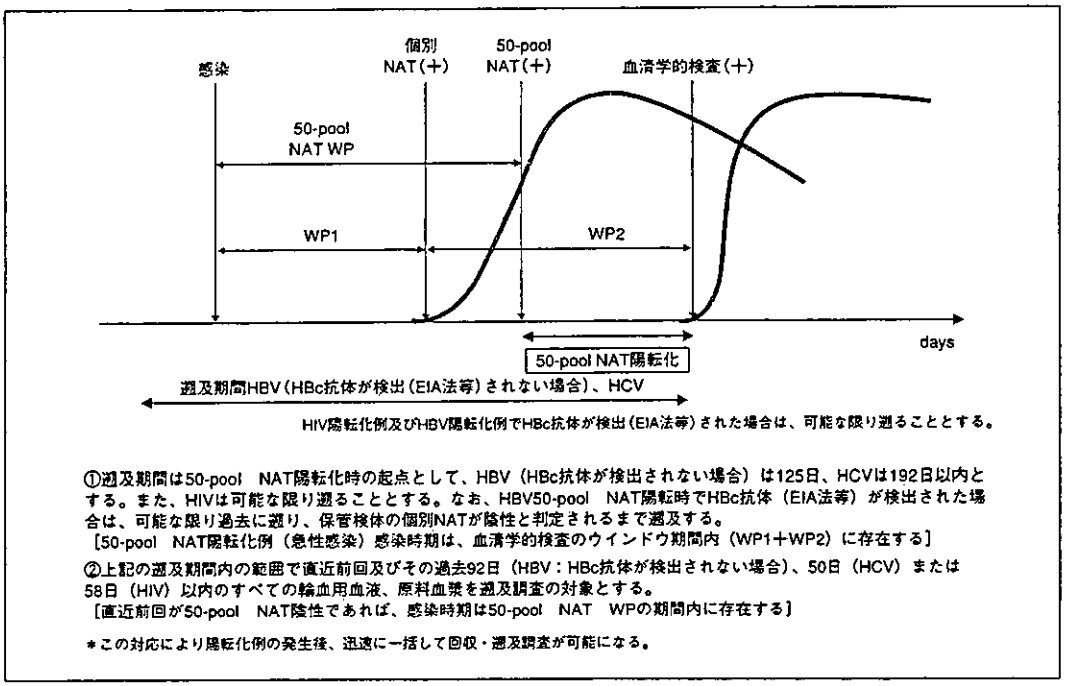
表1

	個別NAT	個別NAT(−)	50プールNAT	50プールNAT(−)	血清学的検査	50プールNAT(+)
	WP	遡及期間	WP	遡及期間	WP	遡及期間
HBV	34日*1	68日	46日	92日	80(44-125)日*2	125日
HCV	23日*1	46日	24.8日	50日	82(54-192)日*1	192日
HIV	11日*1	52日*3	14日	58日*3	22(6-38)日*1	68日*3
梅毒					21-35日*4	35日

遡及期間の設定方法
Schreiberの報告したウインドウ期（WP）は平均値を示すため、個人差による影響およびウイルスの増殖速度を考慮して50プールNAT陰性時期の遡及機関は各WPの2倍の日数とした。また50プールNAT陽性時の遡及期間は血清学的検査のWPの最長期間とする。ただし、HIVについては感染症ウインドウ期間の2倍に感染時期から感染性ウインドウ期間にいたる最大値30日を加算した日数とした。

＊1 Schreiber GB et al. The risk of transfusion transmitted viral infection.N Eng J Med. 1996;334;1685-90.
＊2 50-poolNAT陽性者の追跡調査に基づくRPHAのウインドウ期の推定値
＊3 感染性ウインドウ期を考慮した遡及期間、今井光信、ヒト免疫不全ウイルス、改訂版、日本輸血学会認定医制度指定カリキュラム、日本輸血学会認定医制度審議会カリキュラム委員会編. 2003; 285-288.
＊4 Orton S, Shyphillis and blood donors: What we know, what we do not know, and what we need to know. Transfusion Medicine Reviews 2001; 15;282-91.

（血液製剤調査機構だよりNo 82, 輸血用血液等の遡及調査に関するガイドライン資料より引用）



（血液製剤調査機構だよりNo 82, 輸血用血液等の遡及調査に関するガイドライン資料より引用）

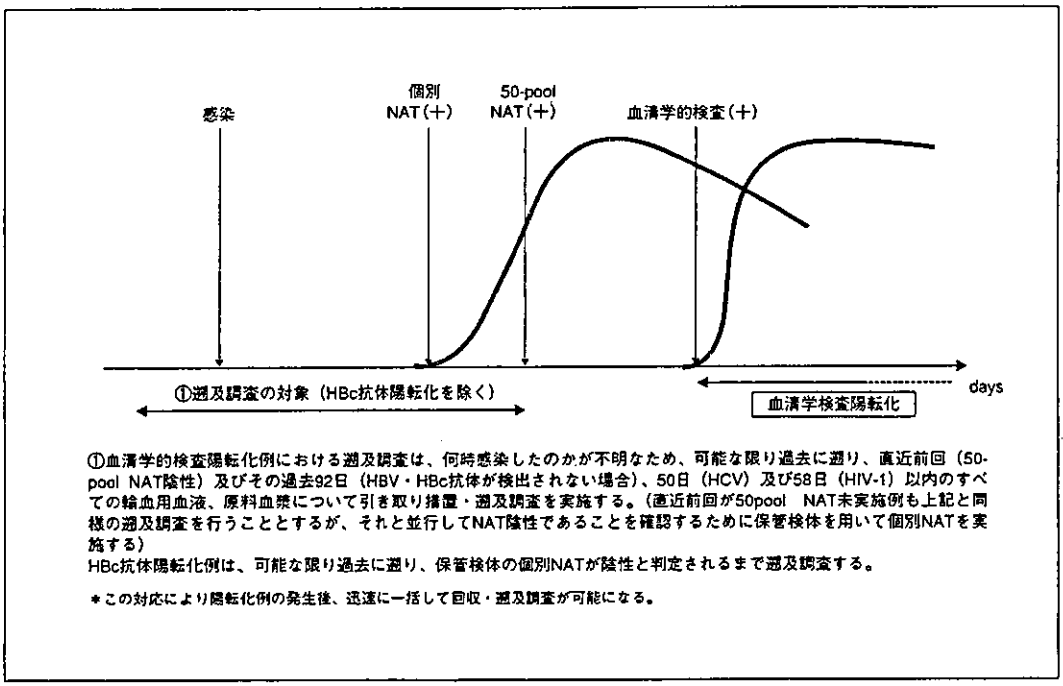
図1．50-pool NAT陽転化例の遡及期間

及することと決められた．

では，血清学的検査での陽転例はどうか（図２）．これにはまず，HBc抗体のみ単独陽性（陽転化）となる例がある．ここで言うところの血清学的陽転化とは，前回の検査まではあらかじめ定めたHBc抗体の基準値を下回っていたのが，今回の検査値で基準値を越えたことを意味する．既に述べたがHBc抗体価の低いドナー（勿論HBsAgは陰性）の血液は感染性は無いと考えられた結果HBc抗体価が日赤の定めた基準値以下の場合には，輸血用血液として昔から採用していた．抗体価が基準値を下回る人の中には，あるときの検査で基準値を上回ることがある（原因は生理的な変動あるいは検査試薬のlot間のわずかな相違などが考えられる）．つまりガイドラインで遡及の対象となるケースの中には真のHBc抗体陽転化ではなく，既往感染を示すHBc抗体価が検出されるが基準値以下であったのがある時の検査で基準値を越える値をとったケースが含まれる．この様な場合にもやはり何時感染したかは特定できないので，可能な限り過去へ遡り，保管検体の個別NATが陰性と判断されるまで遡及することとなった．次にHBs抗原のみ陽転またはHBs抗原とHBc抗体が陽転した場合は，同じく感染時期は特定出来ないのでまず可能な限り遡り，前回および前回からプールNAT空白期間である92日以内の全ての血液製剤，原料血漿について回収措置・遡及を行うこととなった．この遡及ガイドラインは50プールNATを踏まえて捻出されたが，現在は既に20プールNATを行っている．

[2] 新鮮凍結血漿の貯留保管の実施

献血された血液のうち血漿成分の一部はアルブミン等の原料血漿として大量に貯留されたのち，血漿分画センターでアルブミンや第８因子製剤になる．これらはその製造過程で，Solvent/Detergent処理（いわゆるSD処理）[11]が施され，A型肝炎ウイルスやパルボウイルスB19　等のnon-envelope virusを除いて殆どのウイルス（HIV, HBV, HCV等）が不活化される．しかし，献血で得られた血漿の中には，採取後血漿に分離して速やかに凍結されるものがあり，それを新鮮凍結人血漿（Fresh Frozen Plasma（FFP））という．この中には血中の凝固因子が活性を保ったまま存在することから臨床の場でよく使用される．しかし，不活化処理は施されているわけでは無い．従って，ドナーが採血時にウインドウ期間にあった場合には，微量のウイルスが混入していることになる．FFPの有効期間は１年間となっており，もしドナーが１回目の献血から１年以内に再度採血にこなければ，この製剤はスクリーニングの網から漏れて，患者に投与される事になる．言い換えると，１年以内に献血にきて，スクリーニングでウイルス陽性となり，遡及のシステムが機能し，その製剤がまだ未使用であった場合のみ遡及の網にかかる．この解決策として，日赤では，FFPの６ヶ月貯留保管（Quarantine）を行う事にした．２ヶ月間以上貯留保管したFFPの供給は2004年（平成16年）１月から開始しているが，段階的に期間を延長し平成17年７月までには６ヶ月間以上貯留保管したFFPのみを供給する予定となっている．つまり，採血後６ヶ月間はその製剤を出庫しないことにより，遡及が効を奏する可能性をできるだけ高めようということである．果た



（血液製剤調査機構だよりNo 82, 輸血用血液等の遡及調査に関するガイドライン資料より引用）

図２．血清学的検査陽転化例の遡及期間

して，この試みが非常に有効に働くかどうかは懐疑的な意見もあるが，2002年6月から2003年7月までの献血者約580万人中，0.1%が遡及調査対象となっており，うち26.8%が6ヶ月以内に再来していることから，理屈としては，安全性の向上につながると思われる．ちなみに日本で使われる全ての原料血漿については2000年以後6ヶ月間の保管後に使用する体制が整っている．

[3] 輸血用血液の感染性因子の不活化

感染性因子の不活化（Inactivation）技術は，製剤中に混入しているウイルス・細菌などの微生物（あるいはリンパ球等）をあますところなく増殖不能にしてしまう技術であり，血液製剤の安全性の向上への期待は非常に大きい．我が国の血漿分画製剤は，先に述べた様に，S/D処理が施されており，かつ製造工程の種々の段階でウイルスがどれくらい除去されてゆくかがモデルウイルスを使って検討されている．例えば第8因子製剤のクロスエイト製造工程に於けるウイルス不活化・除去効果を，HCVモデルウイルスであるSindbis virus を使って調べるとS/D処理で>5.6log，イムノアフィニティークロマトグラフィーで>5.2log，ナノフィルトレーションで>4.7logで全行程では15.5log以上の不活化・除去効果が期待できる[2]．しかしながら，赤血球製剤，血小板製剤，新鮮凍結人血漿などは，ウイルスの不活化・除去などは施されていない．高感度ウイルス検出法であるNATでも検出不可能な微量の混入ウイルスを不活化することが究極の安全性の確保策として求められている．不活化技術は製剤中の細菌の不活化にも対応している．しかしそれらの短期的，長期的な副作用や製剤の品質への影響についてはまだ検討の余地が残されているようだ．日本赤十字社では，現在欧米で開発されている幾つかの不活化技術のバリデーションを開始したところである．不活化技術としての主流は光増感剤（メチレンブルー，合成ソラレン誘導体S-59[3]，リボフラビン[4]）と紫外線等の照射を組み合わせたいわゆる光不活化技術である．メチレンブルーを用いた新鮮凍結血漿の光不活化[5]はヨーロッパの一部の国（地域）では既に採用しているところもある．その他の不活化技術は，臨床治験を行っているが，実用化にはまだ時間がかかりそうだ．例えばS-59は安全性への懸念が払拭できていないし，リボフラビンは実際に実用化に至るまでにはまだ時間がかかる見通しである．光不活化法は，光を通さない赤血球製剤には不向きである．ウイルスの不活化に当たって光を必要しないS-303[6]やInactine[7]といった物質が開発されている．これらは核酸に直接作用し架橋，修飾するので，赤血球にも応用が可能であるがいずれも治験が中断されていると聞く．従って，現時点では，少なくとも赤血球製剤に関しては，有効な不活化法は存在しないと考えてよい．しかし，人工赤血球の原料としては，赤血球ではなくヘモグロビンを扱うので，むしろ血漿分画製剤に近いものと考えられる．その意味では，赤血球製剤に使用可能な不活化技術は必ずしも必要はないと思われる．

[4] 核酸増幅検査の精度向上

日本赤十字社は1999年10月から数十億円という拠出をして全ての血液製剤に対してNAT検査（HIV, HBV, HCV）の導入に踏み切った．当初は，プール数500で，2000年2月からはプール数50で行っている．すなわちドナー検体を50人づつプールし，その一部を使ってNAT検査を行っている．従って実際には各々が50倍に希釈されている事になる．

輸血後に医療機関から感染があったと報告された件数でみると（つまりスクリーニング検査をすり抜けた可能性があるもの），1994年から2000年までの7年間のNAT未実施期間でHBV感染の医療機関からの自発報告例の中で保管検体等にウイルス核酸が検出された例は31例であり（平均すると年間4-5例，頻度的には11万例に1例），NAT検査開始後は，2001年に5例，2002年に4例，2003年に4例となっており，HBV感染に限って言えばNATは必ずしも劇的な効果を生んでいない．これに対して，HCV感染は57万例に1例，HIV感染は267万例に1例という頻度であったが，NAT開始（1999年）から2004年4月現在でいまだに報告例がない．すなわちHCVとHIVに関しては安全性を高める上で50プールNATは非常に有効であったと言える．つまり空白期間が効果的に短縮できたという事である．HBVで効果がはっきり出ないのはウイルスのdoubling time がHCVより長いことが原因であるとされている．2004年8月から感度を上げる目的で20プールNATが導入されたのでHBVに関しても明らかに有効であるという結果がえられることが期待されている．理想を言えば個別NATが最良であろう．しかし，NATの原理から明らかなように，未知のウイルスや変異ウイルスに対しては対応できない点は念頭に置くべきである．

[5] 複数地域の医療機関の協力を得ての輸血後感染症に関する全数調査の実施

この調査は2004年1月より期間を限定し，医療機関の協力を得て複数の地域で行っているものである．輸血後感染症と確定するためには，輸血前の血液検査で，当該ウイルスに感染していなかったことが証明されなければならない．輸血後肝炎として報告される症例の多くは輸血前の感染症検査を行っていなかったり，不十分であったりする場合が多かった．また，輸血前の検体を保存していないことも多く，調査が不可能な場合もある．そこで，医療機関で患者様にインフォームドコンセントをとり，輸血前と輸血3ヶ月後の血液を採血し保存する．一方，血液センターでは輸血後3ヶ月の血液検体を受け取り，HBV-DNA（血漿分画センター），HCV-RNAとHIV-RNA （血液管理センター）の検査をする．以上のことを医療機関と協力して確実に実施するというものである．検査結果が陰性であれば調査終了．陽性であれば医療機関に保存されている輸血前の検体の当該ウイルスの検査をする．その結果が陽性であれば輸血による感染ではないことになる．結果が陰性であれば，輸血による感染が疑われるので対応する献血者全ての保管検体の個別NATを行い，ウイルスが検出されれば，そのウイルスの核酸の塩基配列を決定し患者様から検出されたウイルスの塩基配列

と比較し因果関係を調べる．もし，保管検体からウイルスが検出されなかった場合には，空白期の可能性があるので献血者の献血以降における感染の有無をフォローアップすることになっている．

[6] E型肝炎ウイルスの疫学的調査の実施

北海道室蘭市内の病院で昨年，心臓手術を受けた60歳代の男性が，輸血が原因でE型肝炎に感染，発病していたことが遺伝子レベルで確認され報告された[8]．輸血によるE型肝炎ウイルス（Hepatitis E virus（HEV））の感染が遺伝子レベルで確認されたのは国内外をとおして初めてである．これを契機として，E型肝炎の疫学的調査が開始されることになった．E型肝炎ウイルスは，おもにアジアの発展途上国で見られる，非A非B型の急性流行性肝炎の病因の一つである．先進国でも散発的な症例報告があるが，その多くは輸入感染と考えられていた．発展途上国では主として経口感染がその主な伝搬ルートと考えられているが，散発的なケースの感染経路はいまだ明らかではなく，その解明は今後の疫学的調査に待たなければならない．周知のとおり，肝炎ウイルスとして，HBVとHCVは輸血後肝炎の原因であり，血液センターのウイルススクリーニングに組み込まれている．血液製剤の安全性の向上は，この2つのウイルスとの戦いであったといってよい．同じく肝炎を発症するA型肝炎ウイルスは，経口感染し感染そのものが一過性であり，輸血による感染が問題とはされていない状況にある．あらたに注目を集めているE型肝炎の致死率は1～2％でA型肝炎の10倍と言われている．また，国内でのIgG抗体保有率は各地域を平均すると5.4％との記載がある[9]．E型肝炎ウイルスは現在のスクリーニングシステムの中には組み込まれていないが，これらのことを考慮すると，日赤での献血者を対象にした疫学調査の結果次第では，今後何らかの形で献血血液のスクリーニングに組み込まれることは十分に考えられる．

[7] 保存前白血球除去の開始

カナダ，イギリス，ドイツ，フランス等の諸外国では保存前白血球除去（Prestorage leukoreduction）を赤血球製剤，血小板製剤，FFP全てについて行っている．国内では1999年6月28日に開催された血液製剤特別部会（現血液事業部会）において導入を進めて行くとされていた．2003年6月4日の血液事業部会での決定をうけて，保存前白徐導入のための本格的な準備がすすめられている．

輸血に伴う様々な有害事象の原因が，血液製剤中に混入している白血球に起因していることが以前より指摘されていた．例えば，1）白血球により血液製剤そのものの品質を低下させる保存障害がおこる（赤血球溶血，凝集，血小板機能低下など），2）Cytomegalovirus（CMV）感染症は，白血球内に存在しているウイルスが原因である，3）同種抗原感作の原因の一つは白血球である，4）Graft versus Host disease（GVHD）は輸注された白血球（リンパ球）が原因で発症する，5）白血球はサイトカインを含めた種々の生物反応修飾物質を遊離するので発熱や蕁麻疹などを誘発する原因ともなりうる．これらのことから病院で血液製剤を使用する場合，白血球除去フィルターが既に広く使用されていたわけである．これがいわゆるベッドサイドフィルトレーションである．しかしながら病院では，全ての輸血製剤について白血球除去（白除）を行っていたわけではなかった．一方，保存前白血球除去は，白血球除去フィルトレーションの操作を血液センターでの採血に引き続いて行うものである．このことにより，全ての血液製剤の中の白血球が一定数以下であることが保証されるようになるとともに白血球に起因すると考えられる有害事象の発生頻度が減少することが期待されるという．しかし，すでに行われているベッドサイドフィルトレーションと比較した場合，保存前に白除することがどれほどの優位性があるかは定かではない．つまり副作用の軽減にどれだけ寄与できるか一定した見解はまだない[10,11]．強いて言えば，白血球に起因する品質の低下が確実に防止できるということであろうか．諸外国で日本より早期に保存前白除が導入された背景には，変異型クロイツフェルド・ヤコブ病が輸血により感染するかも知れないという危惧があった．すなわち当初，プリオンが白血球（Bリンパ球や樹状細胞）を介して伝搬する可能性が指摘されたため白血球の除去がプリオン感染を低減する方法の一つと考えられたのである．現在でも，実験的にはbuffy coatは血漿よりも5-7倍感染性が高いと言われている[12]．

さて，血液製剤1バッグあたりに含まれる白血球数は全血で$1x10^9$個，MAPで$1x10^{8-9}$個，apheresis PCで$1x10^{3-7}$個，新鮮凍結血漿で$1x10^{6-7}$個である．今回の低減化の目標値としては血液製剤1バッグあたり$1x10^6$個以下と定められた（薬事・食品衛生審議会血液事業部会　2003年6月4日）．これに伴ってまずはじめに成分血小板製剤（2004年度），次に成分由来血漿製剤（2005年度），全血採血由来製剤（2006年度）について低減化を計ろうというスケジュール（案）がたてられ現在準備が進められている．

具体的には2004年5月より白血球除去フィルターが装着された採血キットおよびフィルターが無くとも白血球数の低減化が可能な機種についての確認作業がなされているところである．本稿が出来上がるころには白血球除去血小板製剤が出庫されている事と思われる．いずれにしても，白除による効果は，CMVやYersiniaなどの一部の細菌感染症の危険を低減化することが期待できるが，HIVやHCV除去には勿論無効である．その他の副作用の予防効果については今後明らかになって行くであろう．

[8] 献血受け付け時の本人確認の実施への試行

本人確認実施により期待できる効果は，「責任ある献血」の推進と遡及調査の確実性の向上がある．献血者の中には，感染症の検査のために献血したり，自分の住所を偽ったり，他人になりすまして献血する人が少数ながら存在する．献血時に本人確認を実施することでこのような献血者の来所を減少することができると思われる．

例えば，2002年度厚生労働省研究報告「献血者・妊婦に関す

る研究」の結果では，検査サービスを希望された献血者のうち，通知結果が返送されてきた献血者（すなわち住所を偽った献血者）は通知結果が配送された献血者より感染症マーカー陽性率が数倍高い結果となっているという．このことは，遡及不可の例も高くなることを意味している．試行の段階では，証明書提示拒否は全体の１％程度に留まったということから，今後全国的な展開があるものと予想される．

以上，血液事業の展開における新しい動きについて，特に昨年から，その取り組みを宣言している８項目の血液製剤の安全性強化対策について概要を解説した．しかしながらこれらの取り組みを実行するにためにはそれなりの費用がかかることも忘れてはならない．現在の日本の血液製剤の安全性について言えば，NAT検査の導入により非常に高い水準に達しているのである．既に述べたように，NAT開始前ですら，輸血によるHBV感染の頻度は11万例に１例（0.0009％）であった．50プールNAT検査の導入でさらに低下している（HIV, HCVでは今までのところ０％である）．今後莫大な費用をかけて安全性の向上に取り組んだとしても高々0.0009％の向上が見込まれるのみである．つまりcost-effectivenessを念頭に置いて安全性の向上を考えることが重要である．試算によれば，８項目の取り組みをすべて完結するためには数百億円はかかると言われている．血液の安全性を高める技術の進歩はめざましいものがあるが，一方でそれを導入するための費用の捻出が困難な状況になっているのである．こうした問題の一方で，高齢化社会を目前として今後はドナー不足が深刻な問題となりつつある．Dr. Holland（元米国BloodSource Medical Director）がいみじくも "The safest unit of blood could kill you"[13] と述べているように最も安全な血液を求めるあまり，供給可能な血液そのものが不足してしまうというパラドックスもありうることを忘れてはならないと思う．

まとめ

既に述べたとおり，現在日本で開発されているリポソーム包埋型人工赤血球の酸素運搬を担っている部分はヒト由来ヘモグロビンであることから，その安全性確保については，輸血用血液製剤と類似の考え方が必要となるだろう．一方，言うまでもないが，血液型不適合輸血を心配することなく，緊急時に使用でき，かつ長期保存可能であることは，人工赤血球が持つ極めて大きな利点であり魅力である．それ故に，人工赤血球が種々のハードルを越えてヒト赤血球製剤の代替物として臨床現場で使用される日が遠からず訪れることを願っている．

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CONSTRUCTION OF EXPRESSION SYSTEM FOR HUMAN α_1-ACID GLYCOPROTEIN IN *PICHIA PASTORIS* AND EVALUATION OF ITS DRUG-BINDING PROPERTIES

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ABSTRACT:

Human α_1-acid glycoprotein (hAGP) is a plasma glycoprotein that functions as a major carrier of basic ligands. This is the first report of the recombinant hAGP (rhAGP). In this study, rhAGP was expressed in the methylotropic yeast *Pichia pastoris* (GS115) using the expression vector, pPIC9, and then purified by anionic exchange, hydrophobic interaction, and gel filtration chromatography. The molecular weight of rhAGP was much lower than that of hAGP, because of the difference in glycan chain content. Results of glycopeptidase F digestion suggest that the peptide moiety of rhAGP was the same as that of hAGP. The results of circular dichroism spectra measurement indicated that rhAGP predominantly formed a β-sheet-rich structure that was the same as that of hAGP and typical of the lipocalin family. From the experiments using AGP-binding drugs (chlorpromazine, warfarin, and progesterone) and quinaldine red as a probe for the binding site, it was indicated that rhAGP also had the same ligand-binding capacity and binding site structure as hAGP. These findings strongly suggest that this recombinant hAGP (rhAGP) is very useful for the exploration of the ligand-binding site and biological function of hAGP.

Human α_1-acid glycoprotein (hAGP), a member of the lipocalin family, is a polypeptide with two disulfide bonds and five carbohydrate chains that account for about 40% of the total mass of 36 kDa (Halsall et al., 2000). Although the three-dimensional structure and biological functions of hAGP are still unknown, circular dichroism (CD) measurement (Aubert and Loucheux-Lefebvre, 1976) and molecular modeling (Rojo-Dominguez and Hernandez-Arana, 1993) have revealed that it has a predominantly β-sheet structure in aqueous solution. Furthermore, the common structure of the lipocalin protein fold is now well described (Flower et al., 2000). The lipocalin fold is a highly symmetrical all-β-sheet structure dominated by a single eight-stranded antiparallel β-sheet closed back on itself to form a continuously hydrogen-bonded β-barrel.

The main activity of hAGP is the binding of basic drugs and steroid hormones in plasma (Kremer et al., 1988; Baumann et al., 1989; Treuheit et al., 1992). It is very important to evaluate the binding site on hAGP molecules to understand the pharmacokinetics of these drugs. Previous studies have proposed that several amino acid residues were involved in these binding sites (Kremer et al., 1988; Halsall et al., 2000; Kopecky et al., 2003), but their detailed positions are not clear. These residue positions have not been examined using mutants, mainly because of the lack of an established hAGP expression system. Dente et al. (1988) reported the expression of rhAGP in the cultured cell line and in transgenic mice, but there was no evidence of purified rhAGP.

In the present study, we used the methylotropic yeast *Pichia pastoris* as the expression host (Sreekrishna et al., 1988; Cregg and Higgins, 1995; Romanos, 1995) for construction of the hAGP expression system, because of its ability to grow to very high cell density (Cregg and Higgins, 1995) while producing alcohol oxidase at up to 30% of its total soluble protein when fully induced (Cregg et al., 1989); it also secretes very little of its own native protein, simplifying purification of heterologous secreted protein (Barr et al., 1992). Furthermore, *Escherichia coli* and mammalian cells may not be suitable for the AGP expression system for the following reasons. 1) Carbohydrate chains of hAGP have an important role in aqueous solubility. However, *E. coli* can produce only nonglycosylated protein because it lacks an endoplasmic reticulum and Golgi apparatus. 2) In mammalian cells, quantities of recombinant proteins expressed are generally less than in *P. pastoris*.

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Materials and Methods

Materials. hAGP, chlorpromazine, and progesterone were purchased from Sigma-Aldrich (St. Louis, MO). Potassium warfarin was donated by Eisai Co. (Tokyo, Japan). Restriction enzymes, *E. coli* JM109, the DNA ligation kit and the DNA polymerase *Premix Taq* (EX *Taq* version), and glycopeptidase F (GPF) were obtained from Takara Biotechnology Co. (Kyoto, Japan). The DNA sequencing kit was obtained from Applied Biosystems (Tokyo, Japan). The *Pichia* expression kit was purchased from Invitrogen (Carlsbad, CA). DEAE Sephacel, Phenyl Sepharose Fast Flow, and Sephadex G-75 superfine were purchased from Amersham Biosciences Inc. (Piscataway, NJ).

Strain and Plasmid. *E. coli* JM109 was used as the host strain for constructing hAGP/pPIC9. pPIC9 contains the alcohol oxidase I promoter, His^+ selectable marker, and prepro-α-mating factor secretion signal derived from *Saccharomyces cerevisiae*. hAGP cDNA (AGP-A gene) was a gift from Kyowa Hakko Co. (Tokyo, Japan). *P. pastoris* (GS115) was selected as the host strain for expression (Cregg et al., 1989).

ABBREVIATIONS: AGP, α_1-acid glycoprotein; hAGP, human AGP; rhAGP, recombinant hAGP; CD, circular dichroism; GPF, glycopeptidase F; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; MD, minimal dextrose; MM, minimal methanol; Mut, methanol utilization; Mut^+, high methanol utilization; Mut^S, slow methanol utilization; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

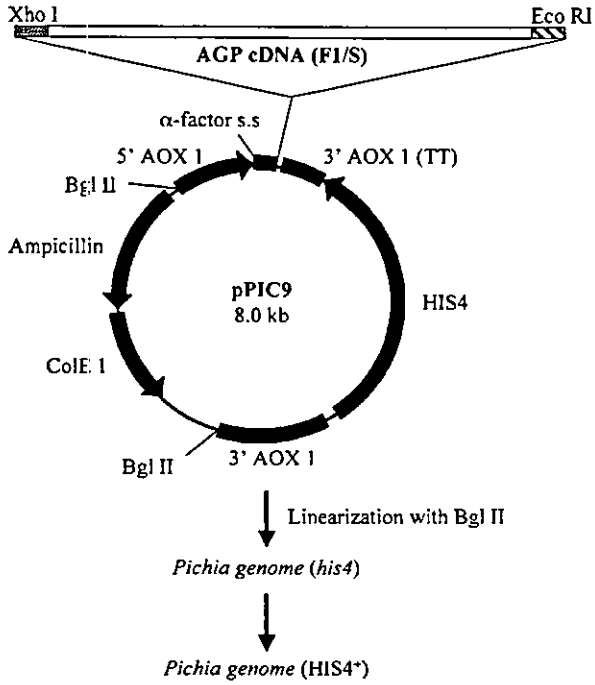

FIG. 1. Expression construct for rhAGP in *Pichia pastoris* (GS115).

Medium. *E. coli* JM109 was grown in Luria broth (Ausubel et al., 1990) containing ampicillin (50 mg/ml). Buffered glycerol-complex medium (BMGY; 1% yeast extract, 2% peptone, 0.1 M potassium phosphate, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol; pH 6.0) and buffered methanol-complex medium (BMMY; same as BMGY except that 0.5% methanol was used instead of glycerol) were used for growing *P. pastoris* and producing rhAGP, respectively. Minimal dextrose (MD) agar (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% dextrose) was used for screening of His^+ transformants, and minimal methanol (MM) agar (same as MD except that 0.5% methanol was used instead of dextrose) was used for methanol utilization (Mut) screening.

Construction of Expression Vector. A 549-base pair DNA fragment encoding hAGP was amplified by PCR using hAGP cDNA (template) and the following oligonucleotide primers: 5' GGACTAGTCTCGAGAAAAGACA-GATCCCATTGTGTGCC-3 (5' XhoI) and 5' GCGGAATTCCTAGGATTC-CCCCTCCTCCTG-3 (3' EcoRI). The PCR reaction mixture (final volume, 50 μl) contained the following: 50 ng of template, 1 μl of primers (20 pmol), 25 μl of *Premix Taq* (0.05 unit/μl) containing 4 mM Mg^{2+}, and 0.4 mM deoxynucleotide. The mixture was subjected to denaturation at 96°C for 5 min and 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, using a 9600 DNA Thermal Cycler (PerkinElmer Life and Analytical Sciences, Boston, MA). The PCR product was purified using a QIAGEN kit (QIAGEN, Valencia, CA), and after digestion with XhoI and EcoRI, it was cloned into the XhoI and EcoRI sites of pPIC9, generating hAGP/pPIC9 (Fig. 1). Portions of hAGP/pPIC9 were sequenced using a 5' XhoI primer.

Transformation of *P. pastoris*. *P. pastoris* GS115 (His^-) was transformed with hAGP/pPIC9 digested with BglII. Approximately 20 μg of linearized plasmid DNA was used for electroporation in 0.2-cm cuvettes, using a Gene Pulser (Bio-Rad, Hercules, CA) at 1.5 kV, 25 μF, and 200 W. Immediately after pulsing, 1 ml of cold 1 M sorbitol was added to the cuvettes. Cells were plated onto MD agar for the selection of His^+ transformants. To screen for methanol utilization, each colony on the MD plate was first spotted onto MM agar and then onto a new MD plate. After 48 h, Mut^S and Mut^+ colonies were identified.

Screening for rhAGP. Colonies for selection of GS115/Mut^S and Mut^+ [hAGP/pPIC9] were inoculated from MD plates to 5 ml of BMMY and incubated for 3 days at 30°C with shaking. Methanol was added every 24 h to a final concentration of 0.5%. Secretion of rhAGP into the culture medium was monitored using 12.5% SDS-PAGE and Coomassie Blue staining. hAGP was used as a reference standard.

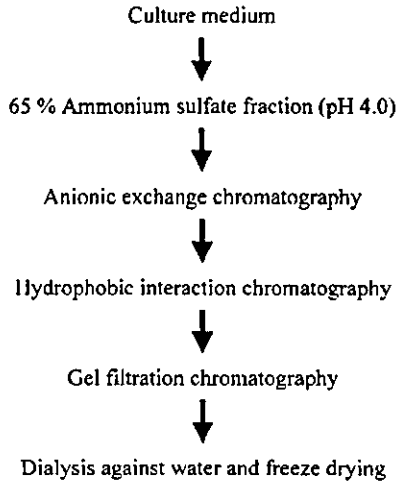

FIG. 2. Flow chart for purification of rhAGP.

Expression of rhAGP. In the growth phase, a Mut^S colony was proliferated in 100 ml of BMGY in a 1-liter flask at 30°C with shaking. In the induction phase, the growth-phase cells were harvested by centrifugation (1500*g*, 10 min, 20°C), and cell pellets were resuspended in 1 liter of BMMY in a 3-liter flask at 30°C with shaking. The cells were then grown for an additional 96 h. Methanol was added to a final concentration of 0.5% every 24 h to maintain induction.

Purification of rhAGP. The growth medium was separated from the yeast by centrifugation (6000*g*, 10 min, 4°C), and the secreted rhAGP was isolated from the medium as follows (Fig. 2). The medium was brought to 65% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4°C, and the pH was adjusted to 4.0. After shaking for 12 h, the precipitated protein was collected by centrifugation (12,000*g*, 60 min, 4°C) and resuspended in distilled water. Dialysis was performed for 48 h at 4°C against 100 volumes of distilled water, followed by a further 24 h of dialysis against 100 volumes of 10 mM Tris-HCl buffer (pH 7.4). Then, the solution was loaded onto a column of DEAE Sephacel. rhAGP was eluted with a linear gradient of 0 to 1 M NaCl in 10 mM Tris-HCl buffer (pH 7.4). The eluted rhAGP was loaded onto a column of Phenyl Sepharose Fast Flow. Finally, rhAGP was purified using Sephadex G-75 superfine.

CD Measurement. The CD spectra were measured using a Jasco Model J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 25°C. The data were expressed as mean residue ellipticity, $[\theta]$. The protein concentration was 0.5 mg/ml for the far-UV CD measurements in 20 mM sodium phosphate buffer (pH 7.4). Cells with 1-mm and 10-mm lightpaths were used for the far- and near-UV CD measurements, respectively.

Western Blot analysis. rhAGP was subjected to 12.5% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. Immunoreactive protein was detected using polyclonal antibodies against hAGP raised in Japanese white rabbits (Tissot et al., 1990). Primary antibody/antigen complexes were conjugated to horseradish peroxidase and developed using the Bio-Rad HRP substrate.

Deglycosylation of hAGP and rhAGP. The deglycosylation of hAGP and rhAGP was performed using GPF. After denaturing with 1% SDS and 2-mercaptoethanol, 25 mg of denatured AGPs were incubated with 1 mU of GPF at 37°C and pH 8.6 for 12 h. Treated and untreated proteins were analyzed by electrophoresis.

Fluorescence Measurement of Quinaldine Red. Fluorescence of quinaldine red was measured using a Jasco FP-770 fluorometer. AGP was dissolved at 10 μM in appropriate buffers. For the measurements of quinaldine red fluorescence, the excitation wavelength was 495 nm, and emission was monitored from 550 to 650 nm. Quinaldine red concentration was varied between 1 and 30 μM in AGP solution (10 μM), because it was reported that hAGP had a single binding site for quinaldine red, which was also the site for hAGP-binding drugs (Imamura et al., 1993, 1994). Spectra were recorded immediately after mixing.

Ligand Binding Assay. Drug-binding parameters were calculated using the

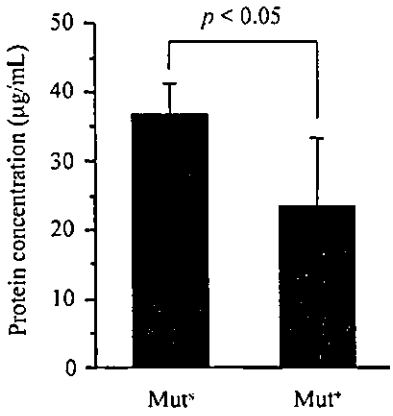

FIG. 3. Screening of secreted colonies (Mut^S and Mut^+).

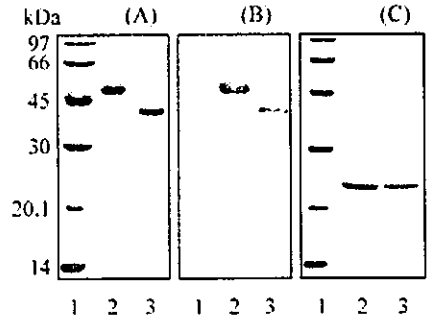

FIG. 4. SDS-PAGE and Western blot analysis of hAGP (lane 2) and rhAGP (lane 3). A, SDS-PAGE of hAGP and rhAGP. B, Western blot analysis of hAGP and rhAGP using hAGP polyclonal antibody. C, SDS-PAGE of hAGP and rhAGP treated with GPF. Lane 1 represents a molecular weight marker.

tryptophan fluorescence quenching method (Nishi et al., 2002). We obtained a fluorometric titration curve by plotting the tryptophanyl fluorescence intensity of AGP (excitation = 295 nm) using a Jasco FP-770 fluorometer. The drug concentration was varied between 0.1 and 60 μM in 2 ml of AGP solution (10 μM). The fluorescence intensity was corrected for the change in AGP concentration with the change in volume. Based on the fluorometric titration curve, straight lines were drawn to represent the lowest and highest concentrations of the drug. The intersection point of these lines was used to obtain the number of binding sites and the binding constant, using eqs. 1 to 6:

$$[P_f] + n[D_f] \leftrightarrow [PD_n] \quad (1)$$

$$K_a = [P \cdot D_n]/n[D_f][P_f] \quad (2)$$

$$[P \cdot D_n] = \Delta Q_f[P_t] \quad (3)$$

$$[P_f] = [P_t](1 - \Delta Q_f) \quad (4)$$

$$n[D_f] = [D_t - n\,\Delta Q_f[P_t] \quad (5)$$

$$K_a = \Delta Q_f/([D_t] - n\,\Delta Q_f[P_t])(1 - \Delta Q_f) \quad (6)$$

where $[P_t]$ and $[P_f]$ are the concentrations of total and free protein, respectively, $[D_t]$ and $[D_f]$ are the concentrations of total and free drug, respectively, n is the number of binding sites, and ΔQ_f is the quenching fraction.

Results

Construction of pPIC9 Expression Vector. To secrete rhAGP in culture medium, hAGP cDNA was inserted into a multicloning site on pPIC9 expression vector (Fig. 1). Heterologous proteins fused to the downstream signal sequence (α-factor sequence) are cleaved in the sequence Glu-Lys-Arg-X by the KEX 2 endopeptidase, which cleaves on the carboxyl side of dibasic residues (Julius et al., 1984). In *S. cerevisiae*, it has been observed that the Glu-Ala repeats adjacent to the KEX 2 cleavage site are not necessary for cleavage by *KEX2* (Brake et al., 1984).

Screening of Secreting Clones. For transformation of hAGP/pPIC9 into *P. pastoris*, the plasmid was linearized by digestion with BglII. His^+ transformants (GS115/His^+) that appeared on the MD plate were spotted onto a MM plate to score for Mut^+ and Mut^S. For protein concentration analysis and electrophoresis, 20 colonies of Mut^+ and 10 colonies of Mut^S, respectively, were monitored in 5 ml of BMMY at 30°C for 3 days. Average protein concentration of Mut^S were higher than that of Mut^+ (Fig. 3). In electrophoresis of Mut^S, bands other than rhAGP were very faint (data not shown). Based on these results, the Mut^S clone that expressed rhAGP most abundantly was selected for large-scale culture.

Physical Characterization. rhAGP was purified using anionic exchange (DEAE Sephacel), hydrophobic interaction (Phenyl Sepharose Fast Flow), and gel filtration (Sephadex G-75 superfine) chromatography. Although results of SDS-PAGE and Western blotting showed a single band, its molecular weight was different from that of hAGP (Fig. 4, A and B). This difference seemed to derive from the difference in molecular weight per glycan chain of each AGP molecule, because it has been reported that most foreign protein secreted from *P. pastoris* is not subjected to extensive glycosylation (mannosylation). Furthermore, it was observed that bands for rhAGP and hAGP treated with GPF appeared at the same position, indicating that the peptide moiety of rhAGP was identical to that of hAGP (Fig. 4C).

Structural Characteristics. Conformational structure of rhAGP was evaluated by the far- and near-UV CD spectra. The far-UV CD spectrum of rhAGP at pH 7.4 and 25°C had a minimum at 217 nm, consistent with the abundance of β-sheet structure (Fig. 5A). On the other hand, the near-UV CD spectrum for rhAGP generated the significant decrease of CD intensity without changing the spectrum pattern compared with that of hAGP (Fig. 5B). These results suggested that rhAGP has formed the same secondary structure as that of hAGP, and the difference in type of glycan chain resulted in the minor change of the tertiary structure of rhAGP.

Ligand-Binding Characteristics. It is known that hAGP has the binding sites for acidic and basic ligands and steroid hormones, respectively, and these sites overlap each other (Maruyama et al., 1990). To investigate the binding capacity of rhAGP to three drugs, chlorpromazine, warfarin, and progesterone, the values of n, the number of binding site, and K_a, association constant, were calculated using the tryptophan fluorescence quenching method (Nishi et al., 2002). Figure 6 shows the titration curves of tryptophanyl fluorescence intensity using chlorpromazine as a typical example. Significant differences in drug-binding properties of these three drugs between hAGP and rhAGP were not observed (Table 1). This result indicated that a ligand-binding capacity of rhAGP was similar or equivalent to that of hAGP.

It has recently been shown in our laboratories that quinaldine red binds strongly and selectively to hAGP and then emits the fluorescence (Imamura et al., 1993, 1994). Therefore, to obtain preliminary information on the binding site on rhAGP, we examined the effect of chlorpromazine, warfarin, and progesterone on the fluorescence of quinaldine red bound to hAGP and rhAGP (Fig. 7). As shown in Fig. 7, in both hAGP and rhAGP, all drugs caused significant decreases in the fluorescence of quinaldine red in the order chlorpromazine > warfarin > progesterone. These results indicated that rhAGP had almost the same drug-binding site structure as that of hAGP.

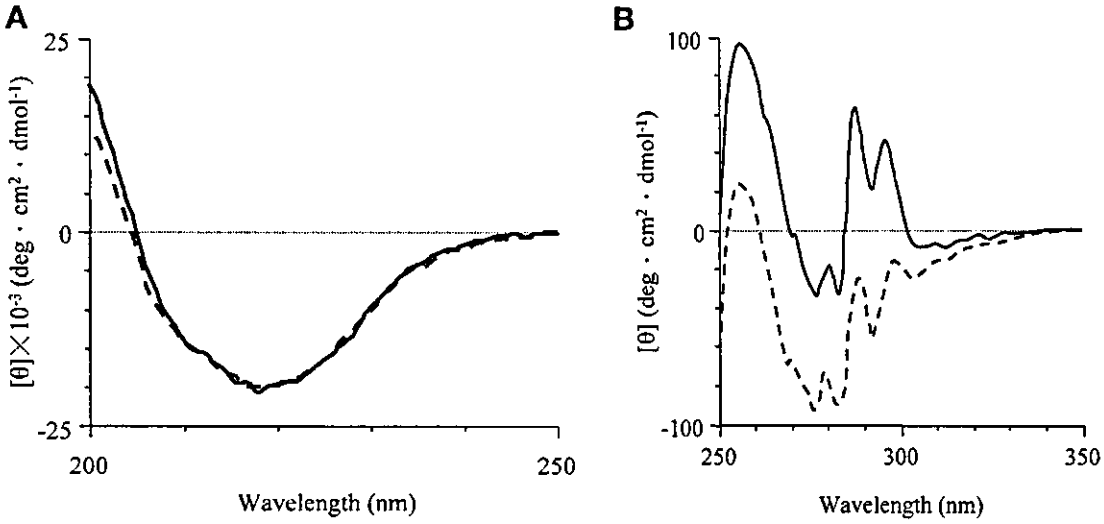

FIG. 5. Far- (A) and near- (B) UV CD spectra of hAGP (solid line) and rhAGP (dashed line).

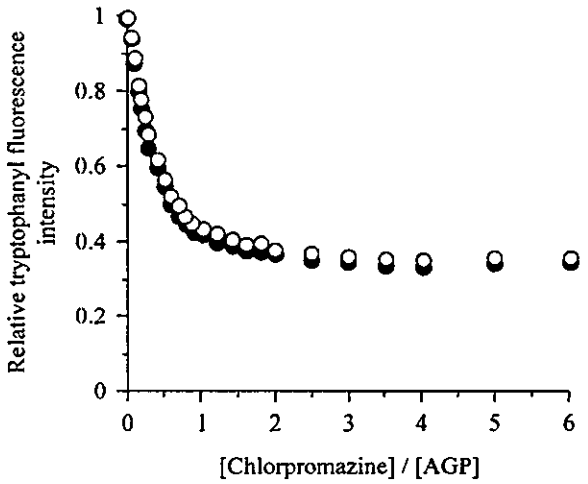

FIG. 6. Quenching of tryptophanyl fluorescence of AGP by chlorpromazine. Symbols represent hAGP (filled circles) and rhAGP (open circles), respectively.

Discussion

hAGP is a plasma protein that contains 183 amino acid residues, five glycan chains, and a largely β-sheet structure in aqueous solution (Halsall et al., 2000). This structural pattern is common to proteins in the lipocalin family (Flower et al., 2000), but detailed structure and biological functions remain obscure.

Of the numerous activities of hAGP that have been described, the most common is binding of ligands, particularly therapeutic drugs. For example, hAGP binds thalidomide and thereby affects the drug's immunomodulatory activity against tumor necrosis factor-α (Turk et al., 1996). hAGP is reportedly involved in intracellular events, such as controlling thrombocytic agglutinability, controlling bacterial englobement, extension during engrafting, and inhibiting lymphocyte growth (Kremer et al., 1988; Baumann et al., 1989; Turk et al., 1996). Although many functions of hAGP are reported, it is not known which part of the hAGP molecule is involved in each function. One of the major reasons for this is the lack of an established rhAGP expression system.

In this study, we constructed the first hAGP expression system that uses the yeast *Pichia pastoris*. Two phenotypes, Mut^S and Mut^+, were observed in the screening process after linearized hAGP/pPIC9 digested with BglII was inserted into *P. pastoris* (GS115) by a single crossover event. From the level and pattern of rhAGP expression, Mut^S strains were selected, although it has been reported that Mut^+ strains of *P. pastoris* produce high levels of biomass in fermentation and, for *S. cerevisiae*, secretion is localized to the growing bud tip (Schekman and Novick, 1982; Digan et al., 1989).

The prepro-α-mating factor leader sequence derived from *S. cerevisiae* allows effective secretion and processing of rhAGP in *P. pastoris*. Cleavage of the leader is apparently mediated via KEX 2 activity in *P. pastoris*. In the secretory pathway of yeast, the signal peptide is removed by a peptidase (Blobel, 1977), and folding occurs in the endoplasmic reticulum with the assistance of accessory proteins including disulfide isomerase (Freedman, 1989). *P. pastoris* seems to have an advantage in the glycosylation of secreted proteins in that it does not appear to hyperglycosylate proteins, unlike *S. cerevisiae*. Both yeasts mainly produce *N*-linked glycosylation of the high-mannose type. However, the length of the oligosaccharide chains added post-translationally to proteins is much shorter in *P. pastoris* (average, 8–14 mannose residues per side chain) than in *S. cerevisiae* (50–150 mannose residues) (Grinna and Tschopp, 1989), and very little *O*-linked glycosylation has been observed in *P. pastoris*.

In the experiments for the drug-binding function, the order in the degree of the quenching of tryptophanyl fluorescence (data not shown) and displacement of quinaldine red for hAGP and rhAGP was as follows: chlorpromazine > warfarin > progesterone. This could be due to a slight difference in the binding region of the drugs, since rhAGP has almost the same binding capacity and number of binding sites as that of hAGP (Table 1). The binding region of chlorpromazine may be in the neighborhood of tryptophan residues and may overlap with that of quinaldine red to an extent greater than that between quinaldine red and progesterone. Since quinaldine red and chlorpromazine belong to the basic drug group, they may interact with hAGP and rhAGP at the basic drug-binding region.

Whereas there are some reports that oligosaccharide chain and sialic acid influence the structure of hAGP (Sebankova et al., 1999) and the binding of some drugs to hAGP (Friedman et al., 1986; Shiono et al., 1997), our data indicate that the types of glycan chain attached to hAGP and rhAGP do not greatly affect its ligand-binding properties, despite minor changes of tertiary structure. It is known that some biological functions of hAGP were strongly linked to glycoform, including sialic acid (Sialyl $Lewis^x$) (Fournier et al., 2000), and

TABLE 1

Comparison of ligand-binding capacity between hAGP and rhAGP

Three ligands were used: warfarin, chlorpromazine, and progesterone. Data are presented as mean ± standard deviation. n and K_a (10^6) represent the number of binding sites and association constant, respectively.

	Chlorpromazine		Progesterone		Warfarin	
	n	K_a	n	K_a	n	K_a
hAGP	0.98 ± 0.04	1.53 ± 0.57	1.03 ± 0.06	2.73 ± 0.40	1.01 ± 0.10	3.65 ± 1.23
rhAGP	0.99 ± 0.07	2.16 ± 1.35	1.00 ± 0.12	1.83 ± 0.32	0.95 ± 0.04	3.04 ± 0.56

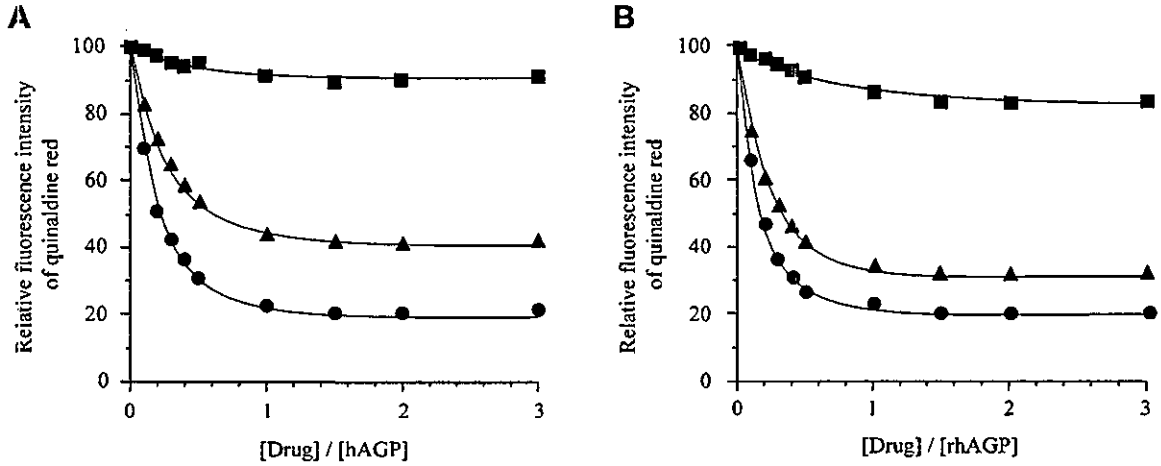

FIG. 7. Displacement of quinaldine red by AGP-binding drug. The experiments were performed at a quinaldine red concentration of 10 μM, and hAGP (A) and rhAGP (B) concentration of 10 μM. Drugs used in this experiment were as follows: progesterone (squares), warfarin (triangles), and chlorpromazine (circles).

others to peptide moiety (Boutten et al., 1992; Van Molle et al., 1997). Therefore, rhAGP, studied here, is fully used for drug binding studies, but it may or may not be used for the investigation of the biological functions.

The present results indicate that rhAGP produced in *P. pastoris* is very useful for evaluation of structural and functional properties of hAGP. Therefore, mutants prepared using this expression system may bring a lot of information about structural and drug-binding properties. However, in most individuals, hAGP exists as a mixture of two or three genetic variants: A variant and the mixture of F1/S variant, and more than 20 substitutions of amino acid residues between these variants were found (Yuasa et al., 1987; Eap and Baumann, 1989). It has been reported that there are differences in the binding capacity of some ligands between these hAGP variants (Herve et al., 1998), although the structure of these variants is almost the same in physiological condition (Kuroda et al., 2003). In the present study, we used F1/S variant coded by AGP-A gene, not A variant coded by AGP-B/B′, for expression of hAGP because, in blood, F1/S variant comprises about 70% of whole AGP (Yuasa et al., 1987; Eap and Baumann, 1989). Of course, the expression of A variant should be investigated, and this study is currently underway.

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Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients

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Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients.

Background. Intravenous iron administration (IVIR) is effective for correcting anemia in hemodialysis (HD) patients. However, it may also enhance the generation of hydroxyl radicals. Recently, plasma proteins have been demonstrated to be extremely susceptible to oxidative stress. Therefore, we investigated the effect of IVIR on the oxidative status of albumin, a major plasma protein, in HD patients.

Methods. Eleven hemodialysis (HD) patients were treated with 40 mg of saccharated ferric oxide intravenously after every dialysis session for four weeks, and 11 age-/gender-matched HD patients were treated with vehicle. We performed high performance liquid chromatography (HPLC) analysis of serum albumin and determined the levels of reduced and oxidized albumin. Carbonyl formation of plasma proteins were also measured using an anti-2,4 dinitrophenylhydrazine antibody in patients with or without IVIR.

Results. IVIR resulted in an increase in both disulfide form (f(HNA-1)) and oxidized form (f(HNA-2)) of albumin in HD patients (36.0 ± 6.03 vs. 41.7 ± 6.27; 5.46 ± 1.50 vs. 8.7 ± 2.22, respectively, $P < 0.05$). The findings here also show that IVIR substantially increased plasma protein carbonyl content by oxidizing albumin. In addition, we found a strong correlation between plasma carbonyl content and the levels of oxidized albumin (f(HNA-1) and f(HNA-2)) in HD patients ($R = 0.674$ and $R = 0.724$, respectively, $P < 0.01$).

Conclusion. The results of this study indicate that the HPLC analysis of serum albumin represents a potentially useful method for the quantitative and qualitative evaluation of oxidative stress in HD patients, and strongly suggest the possibility that oxidative stress, generated by IVIR, enhances the oxidation of albumin in those patients.

[1]These two authors contributed equally to this work.

Key words: albumin, oxidative stress, hemodialysis, saccharated ferric oxide.

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Oxidative stress has long been incriminated in the development of dialysis-related pathologic conditions, such as β_2-microgloblin amyloid arthropathy and accelerated atherosclerosis [1]. Until recently, direct evidence for in vivo oxidative stress in HD patients relied almost entirely on the measurement of lipid peroxidation by-products such as malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS)[2]. Despite the fact that proteins are extremely susceptible to oxidative stress, reports on the detection of oxidatively modified proteins have not been extensive. However, the measurement of the markers for protein oxidation, such as advanced protein oxidation products (AOPP) or carbonyl contents, has recently been applied to assess the oxidative stress in the pathologic conditions. In 2001, Himmelfarb et al [3] reported for the first time that the oxidation of albumin accounts for almost all of the excess plasma protein oxidation in uremic patients as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an immunoassay using an anti-2,4 dinitrophenylhydrazine (DNP) antibody developed by Shacter et al [4, 5]. Human serum albumin (HSA) is a mixture of mercaptalbumin (HMA; reduced form) and nonmercaptalbumin (HNA; oxidized form) [6, 7]. HMA contains one highly reactive sulfhydryl group at position 34 (Cys-34), while other serum proteins have little or none [8]. HNA is composed of at least three types of molecules. The major HNA component is a mixed disulfide with cysteine or glutathione (HNA(Cys) or HNA(Glut))[6, 7]. The other is a more highly oxidized product than the mixed disulfide, such as sulfenic (–SOH), sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) states (HNA(Oxi)), the proportions of which are extremely small in extracellular fluids [9–11]. The high-performance liquid chromatography (HPLC) analysis of albumin developed by Sogami et al permits the clear separation of HSA into HMA and HNA [6], and is used for the determination of the redox state for various pathophysiologic conditions [12–15].

Anemia is a major complication in HD patients, and because of this, most patients routinely receive iron intravenously (IVIR) for anemia correction. Although IVIR has been shown to improve both survival and quality of life of HD patients [16–18], it has been suggested that IVIR may enhance the generation of hydroxyl radicals in the body through the inflammation process and the Fenton reaction [19, 20]. Tovbin et al reported that IVIR in HD patients induced an increase in the level of protein oxidation products, as assessed by AOPP levels, and that this effect is positively related to the inflammatory state of patients [21]. They proposed that IVIR and inflammation synergistically induce oxidative stress. However, the effect of IVIR in HD patients on individual plasma proteins, especially albumin, the most abundant protein, has not been extensively investigated. Therefore, the purpose of this study was to determine the effect of iron and reactive oxygen species on the HPLC profiles of serum albumin in vitro, and then to investigate the effect of IVIR on the oxidation levels in serum albumin in HD patients.

In the current studies we report on the oxidation of albumin by HPLC analysis and the carbonyl content of individual plasma proteins in HD patients with or without IVIR, as well as in healthy subjects. The findings here show that albumin is oxidized to a greater extent in HD patients than in healthy subjects, and that the oxidation of albumin was significantly enhanced in patients receiving IVIR. Since albumin is the major plasma protein target for oxidative reactions, HPLC analysis represents a potential assay method for the assessment of oxidative stress levels in HD patients.

METHODS

Patients

The protocol used in this study was approved by the institutional review board, and informed consent was obtained from all subjects. Twenty-two stable HD patients (15 men, 7 women) aged 25 to 87 years, with a dialysis age ranging between 1 and 9 years, were enrolled in this study. Eleven age- and gender-matched healthy subjects were also investigated as a control group. End-stage renal failure was caused by glomerulonephritis ($N = 8$), nephrosclerosis ($N = 3$), diabetic nephropathy ($N = 9$), and unknown causes ($N = 2$). At inclusion, all patients were on regular bicarbonate hemodialysis therapy (4 to 5 hours three times weekly) using high-flux polysulfone hollow-fiber dialyzers, and had not received IVIR for the last three months. All patients were not treated with antioxidants such as vitamin E and C in the three months before inclusion onto the study. Patients were divided into two groups. The first group (IVIR group) was administered 40 mg of saccharated ferric oxide (Fesin®, Mitsubishi Pharma Co., Ltd., Osaka, Japan) intravenously after every dialysis session for four weeks (total dosage: 480 mg of saccharated ferric oxide). The other group (no IVIR group) was treated with vehicle (saline) for four weeks. At the end of the IVIR or vehicle treatment period, blood samples were obtained before the first dialysis session of the week.

Chromatographic analysis of serum albumin in hemodialysis patients

HPLC was performed as described by Hayashi et al [22]. Serum samples obtained from each patient were immediately frozen and stored at −80°C until used for HPLC analysis. HPLC analysis of 5 μL aliquots of each serum was performed using a Shodex Asahipak ES-502N column (Showa Denko Co., Ltd., Tokyo, Japan; column temperature; 35°C ± 0.5°C). The HPLC system was composed of an intelligent pump L-6200 equipped with a gradient programmer and an F-1050 fluorescence detector (Hitachi Co., Ltd., Tokyo, Japan). Elution was performed by means of a linear gradient with increasing ethanol concentrations from 0% to 5% for serum in 0.05 mol/L sodium acetate and 0.40 mol/L sodium sulfate mixture (pH 4.85) at a flow rate of 1.0 mL/min. From the HPLC profiles of HSA, the value of each albumin fraction (f(HMA), f(HNA-1), and f(HNA-2)) was estimated by dividing the area of each fraction by the total area corresponding to HSA.

Plasma protein carbonyl contents measurement

Plasma protein carbonyl contents were determined by the method of Climent et al [23].

Western blots

The oxidation of individual plasma proteins was measured by Western blot analysis as previously described by Shacter et al [5]. Plasma was diluted to 2 mg/mL of total protein with phosphate-buffered saline (PBS), and derivatized with DNP using an OxyBlot Kit (Serologicals Corporation, Norcross, GA, USA). Samples were diluted to 1 mg/mL of total protein by the addition of an equal volume of nonreducing sample buffer, and 15 μL samples were electrophoresed on duplicate SDS-PAGE gels [24]. Following electrotransfer to a polyvinylidene difluoride (PVDF) membrane, one blot was stained for DNP using the OxyBlot Kit reagents. The second blot was stained with Coomassie brilliant blue G for protein. Bands were visualized with chemiluminescent chemicals and captured on film at 10 minutes.

Analysis of blots

Each Western blot included samples from both patients and healthy subjects so that in all experiments, patient samples could be compared with equivalent data on healthy subjects developed under the same conditions.

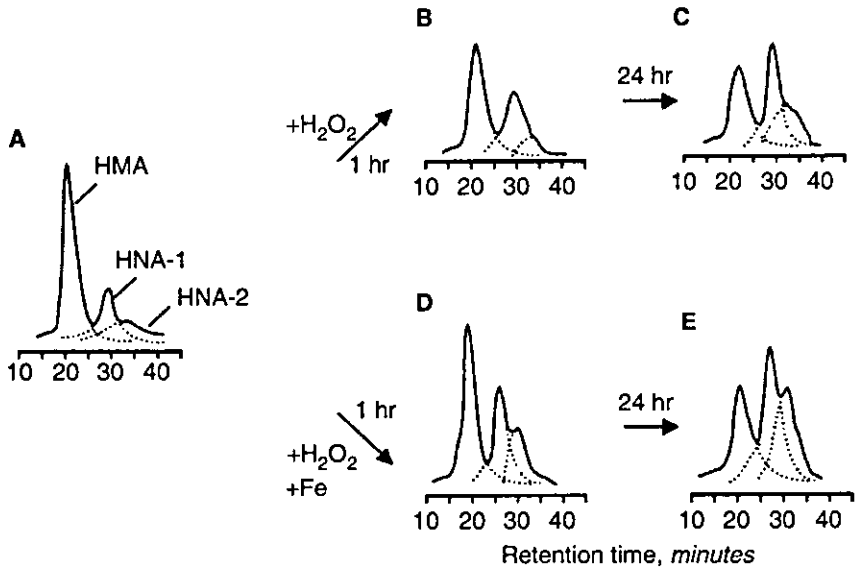

Fig. 1. High-performance liquid chromatography (HPLC) profile of in vitro–oxidized serum albumin. HPLC profile of albumin from a normal subject under basal condition (*A*). Serum was incubated with 200 μmol/L of H_2O_2 in the presence or absence of 180 μmol/L of saccharated ferric oxide for 1 hour (*B* and *D*) or 24 hours (*C* and *E*). Five-μL aliquots of each serum were analyzed by HPLC using a Shodex Asahipak ES-502N column. HMA; mercaptalbumin (reduced form), HNA-1; nonmercaptalbumin (disulfide form), HNA-2; nonmercaptalbumin (oxidized form).

For each experiment, the carbonyl density was determined from the blot with the shortest possible exposure time required to produce clearly visible bands. DNP and protein blots were scanned using the same size section of the blot for each scan. The uniform window size and analysis box ensured that data were being analyzed consistently from band to band and from blot to blot. Because the concentration of various plasma proteins differs between patient groups and healthy subjects, densitometry data for the area of the DNP blot band were divided by the densitometry data for the area of the protein blot band obtained under identical gel loading and electrotransfer conditions. These data are recorded as DNP area/protein area and are reported in densitometry units. The mean for each subject group was calculated from each blot.

Statistics

Statistical significance was evaluated using the two-tailed, unpaired Student *t* test for comparisons between two means, or analysis of variance (ANOVA) analysis followed by Newman-Keuls method for more than two means. A value of $P < 0.05$ was regarded as statistically significant. Results are reported as mean ± SE.

RESULTS

Oxidation of serum albumin by H_2O_2 and Fe in vitro

The current study was designed to determine the redox state of HD patients, and to examine the effect of IVIR on oxidative stress, especially oxidation of serum albumin, in HD patients. First of all, we determined the HPLC profile of serum albumin before and after in vitro oxidation by H_2O_2 and Fe treatment. HPLC analysis allowed us to determine the oxidation status of Cys-34 residues in the albumin. Sera from healthy subjects were incubated with 200 μmol/L of H_2O_2 with or without 180 μmol/L of saccharated ferric oxide for 1 hour or 24 hours, and then subjected to HPLC analysis as described in the **Methods** section. Figure 1 shows typical HPLC profiles of serum albumin from a healthy volunteer before and after oxidation treatment. Serum albumin is separated into three peaks by the HPLC column (Fig. 1A). The first peak represents HMA (reduced form), the second peak, HNA-1 (disulfide form; HNA(Cys) or HNA(Glut)), and the third peak HNA-2 (oxidized from; HNA(Oxi)). Treatment of serum with 200 μmol/L of H_2O_2 for 1 hour resulted in a slight increase in HNA-1 and almost no change in HNA-2 (Fig. 1B). Twenty-four hours after incubation, HMA was reduced and both HNA-1 and HNA-2 fractions were further increased, indicating the oxidation of Cys-34 residues in the albumin (Fig. 1C). In the presence of 180 μmol/L of saccharated ferric oxide, treatment of serum with 200 μmol/L of H_2O_2 further potentiated the oxidation of albumin both at 1 and 24 hours (Fig. 1D and E). The ratio of each albumin fraction to the total albumin (f(HMA), f(HNA-1), and f(HNA-2)) was calculated, and those data are summarized in Table 1. As shown in Table 1, incubation of serum with 200 μmol/L of H_2O_2 and 10 μg/mL of saccharated ferric oxide for 24 hours markedly increased f(HNA-1) and f(HNA-2) values when compared with those with H_2O_2 treatment alone ($P < 0.05$). These results suggest that treatment with saccharated ferric oxide could enhance the oxidation of serum albumin in the presence of H_2O_2, and that HPLC analysis of serum albumin provides quantitative and qualitative values for the oxidation status of albumin.

Oxidation of serum albumin in HD patients

Next, we determined the oxidation status of serum albumin by HPLC analysis from HD patients with or

Table 1. f(HMA), f(HNA-1), and f(HNA-2) values (%) for reduced and oxidized albumin in serum from normal subjects treated with H_2O_2 in the presence or absence of saccharated ferric oxide

		Without saccharated ferric oxide			With saccharated ferric oxide		
		0 hr	1 hr	24 hrs	0 hr	1 hr	24 hrs
	f(HMA)	69.5 ± 0.2	67.3 ± 0.7	45.9 ± 1.1	69.9 ± 3.5	62.3 ± 0.8	44.9 ± 0.8
H_2O_2	f(HNA-1)	24.9 ± 0.7	26.6 ± 1.6	35.0 ± 1.4	23.0 ± 0.5	27.7 ± 0.6	45.5 ± 0.4[a]
(200 μ mol/L)	f(HNA-2)	5.6 ± 0.9	6.1 ± 1.7	8.5 ± 2.5	7.1 ± 2.0	9.7 ± 0.2	13.6 ± 1.1[a]

[a] $P < 0.05$ as compared with serum treated with H_2O_2 in the absence of saccharated ferric oxide. Values are expressed as mean ± SE ($N = 4$).

Table 2. f(HMA), f(HNA-1), and f(HNA-2) values (%) for reduced and oxidized albumin in serum from normal subjects and patients without IVIR treated with vehicle or saccharated ferric oxide

	Healthy subjects		Patients without IVIR	
	Vehicle	Saccharated ferric oxide	Vehicle	Saccharated ferric oxide
f(HMA)	66.7 ± 0.7	64.6 ± 0.4	45.4 ± 2.1	36.9 ± 1.0[a]
f(HNA-1)	27.8 ± 0.6	29.7 ± 0.5	41.3 ± 2.3	43.6 ± 1.6
f(HNA-2)	5.5 ± 0.1	5.6 ± 0.2	13.3 ± 0.6	19.5 ± 1.5[a]

[a] $P < 0.05$ as compared with serum treated with vehicle. Values are expressed as mean ± SE ($N = 7$).

without IVIR, as well as from healthy subjects, because albumin is the most abundant serum protein and has been shown to be a target of oxidative stress in uremic patients. Patient profiles of healthy subject group, IVIR group, and no-IVIR group are summarized in Table 2. Data in the patients group were obtained after the treatment period. Parameters for iron status were examined before and after IVIR. Patient groups did not differ in respect to age, gender, diabetes/nondiabetes ratio, duration and efficacy of dialysis, serum albumin, uric acid, and C-reactive protein (CRP) levels. Serum Fe and TSAT levels in HD patients with IVIR were slightly, but not significantly, higher than in those without IVIR, and serum ferritin levels were significantly increased in IVIR group due to iron administration ($P < 0.05$). Figure 2 shows typical HPLC profiles for serum albumin from healthy subjects (Fig. 2A), HD patients without IVIR (Fig. 2B), and with IVIR (Fig. 2C). The ratio of each albumin fraction to the total albumin (f(HMA), f(HNA-1), and f(HNA-2)) was calculated and those data are summarized in Table 3. As shown in Figure 2 and Table 3, f(HMA) was substantially decreased and both f(HNA-1) and f(HNA-2) were significantly increased in HD patients compared with healthy subjects ($P < 0.05$). IVIR treatment resulted in marked increases in both f(HNA-1) and f(HNA-2) in HD patients ($P < 0.05$), while there was no significant change in f(HMA). These findings suggest that Cys-34 residue in albumin is highly oxidized in HD patients and that IVIR resulted in the overoxidation of albumin. In order to determine the relationship between the redox status of plasma proteins and the Cys-34 oxidation of serum albumin, as detected by HPLC, the plasma protein carbonyl contents of these patients were also determined.

Plasma protein carbonyl contents in HD patients

In most cases, protein oxidation is associated with an increase in carbonyl content. It is known that an increase in carbonyl contents reflects the oxidation of Lys, Arg, or Pro residues in a protein. Plasma protein carbonyl contents were significantly increased in HD patients, and IVIR further enhanced the carbonylation of plasma protein (IVIR: 2.2 ± 0.4 nmol/mg protein, $N = 11$; no IVIR: 1.0 ± 0.1 nmol/mg protein, $N = 11$; healthy subjects: 0.4 ± 0.03 nmol/mg protein, $N = 11$, $P < 0.05$). We next determined the carbonyl contents of major plasma proteins separately by Western blot analysis using an anti-DNP antibody. Figure 3A shows a representative blot and Figure 3B summarizes results obtained from the multiple blots. Interestingly, only albumin was significantly oxidized in HD patients, and IVIR increased the albumin oxidation ($P < 0.05$). There was no statistically significant difference in the carbonyl contents of other plasma proteins such as transferrin, immunoglobulin, and fibrinogen among three groups (healthy subjects, HD patients without IVIR, and HD patients with IVIR). These findings suggest that the origin of the increase in plasma protein carbonyl contents in HD patients was largely from an increase in oxidized albumin, and that IVIR substantially increased plasma protein carbonyl contents by oxidizing albumin.

Relationship between HPLC profile of albumin and plasma protein carbonyl content

In order to validate the usefulness of HPLC analysis of serum albumin for the assessment of oxidative stress in HD patients, we determined the relationship between plasma protein carbonyl contents and f(HNA-1) or f(HNA-2) values because plasma protein carbonyl contents are one of the reliable and widely used oxidative stress markers. As shown in Figure 4, both f(HNA-1) and f(HNA-2) have strong correlation with plasma protein carbonyl contents ($R = 0.674$ and $R = 0.724$, respectively, $P < 0.01$). We also investigated the relationship between AOPP levels and f(HNA-1) or f(HNA-2) values, and obtained significant correlation (data not shown). These findings suggest the possibility that f(HNA-2) values can be useful markers for the evaluation of redox status of HD patients.

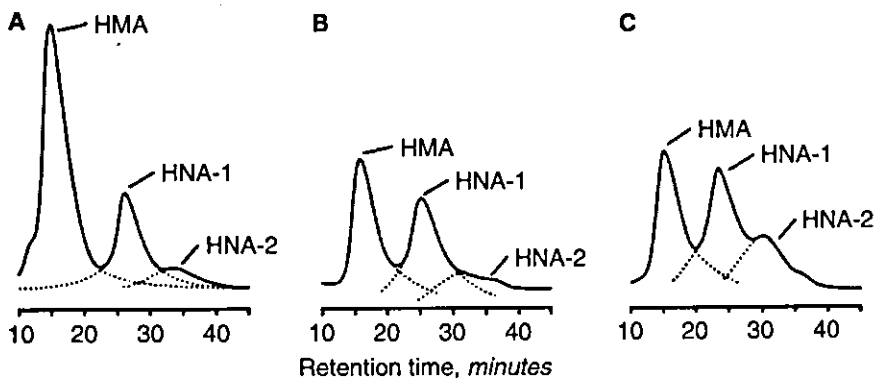

Fig. 2. High-performance liquid chromatography (HPLC) profile of serum albumin from HD patients with or without intravenous iron administration (IVIR). Five-μL aliquots of serum from HD patients with or without IVIR, and healthy subjects were subjected to HPLC analysis using a Shodex Asahipak ES-502N column. A representative tracing of HPLC profiles of serum albumin from normal subjects (*A*). A representative tracing of HPLC profiles of serum albumin from HD patients without IVIR (*B*). A representative tracing of HPLC profile of serum albumin from patients treated with IVIR (*C*).

Table 3. Characteristics of patient group

	Healthy subjects ($N = 11$)	Patients without IVIR ($N = 11$)		Patients with IVIR ($N = 11$)	
		After vehicle	Before vehicle	After IVIR	Before IVIR
Age *years*	67.7 ± 0.4	68.0 ± 3.7		68.6 ± 5.6	
Gender *M/F*	7/4	8/3		7/4	
Duration of dialysis *months*	–	22.4 ± 7.5		22.5 ± 7.1	
Diabetes/nondiabetes	–	5/6		4/7	
Kt/Vurea	–	1.46 ± 0.04		1.52 ± 0.08	
Albumin *g/dL*	4.27 ± 0.07	3.73 ± 0.11		3.53 ± 0.14	
Uric acid *mg/dL*	4.79 ± 0.3	8.1 ± 0.6		7.8 ± 0.4	
CRP *mg/dL*	0.15 ± 0.04	0.28 ± 0.06		0.27 ± 0.08	
Fe μg/*dL*	83.82 ± 5.55	47.6 ± 6.5	49.4 ± 5.8	55.4 ± 2.6	45.5 ± 4.4
TSAT %	28.97 ± 2.61	23.0 ± 6.7	21.2 ± 8.7	28.6 ± 6.2	21.0 ± 1.7
Ferritin *ng/mL*	43.27 ± 4.99	96.9 ± 19.1	102.5 ± 24.3	231.4 ± 55.4[a]	75.4 ± 16.2

[a] $P < 0.05$. Values are expressed as mean ± SE.

DISCUSSION

The current studies demonstrate that serum albumin is highly oxidized in HD patients, with an increase in the disulfide form, and that IVIR on these patients significantly increased the oxidation status of albumin, as evidenced by a marked increase in the oxidized form. To our knowledge, this is the first report demonstrating the effect of IVIR on the oxidation of serum albumin in HD patients. Plasma is known to contain a wide range of important antioxidants, including albumin, ascorbate, and urate. In contrast, the concentrations of superoxide dismutase, reduced glutathione, and catalase, all of which are known to be important intracellular antioxidants, are low in the plasma and are not likely to serve as important plasma antioxidants [25, 26]. It has previously been established that certain amino acid residues of proteins are particularly susceptible to free radical attack [27, 28], and several studies have confirmed that Cys-34 of serum albumin is highly accessible to reactive oxygen species such as H_2O_2 [29, 30] and carbon-centered free radicals [31], as well as other oxidizing agents, such as nitric oxide (NO) [32–34] and peroxynitrite (ONOO−) [29, 35]. In addition, it has been demonstrated that Cys-34 of oxidized albumin is further oxidized to sulfenic, sulfinic, or sulfonic state under stronger H_2O_2-mediated [29, 30] and anaerobic NO–mediated [33] oxidizing conditions. In plasma, free thiol groups are quantitatively the most important scavengers of oxidants, and are known to be largely located on the albumin molecule. Furthermore, we confirmed the carbonyl formation of plasma albumin, as previously demonstrated by Himmelfarb et al [3], suggesting that basic amino acid residues in albumin as well as Cys-34 are also oxidized. Because albumin is the most abundant plasma protein, it could play a major role as an antioxidant in plasma at least by thiol oxidation and carbonyl formation. We recently demonstrated that the plasma half-life of radiolabelled- and in vitro oxidized-albumin was substantially decreased in mice, and that the liver uptake clearance of oxidized albumin was markedly increased by 11-fold [36]. These findings suggest that serum albumin might play an important protective role against reactive free radicals in extracellular fluids via its oxidation and the subsequent clearance from the systemic circulation by the liver. In this context, we expected that the characterization of oxidation status of serum albumin might provide useful information regarding the redox state of the human body, prompting us to examine the effect of IVIR on the oxidation of albumin. As shown

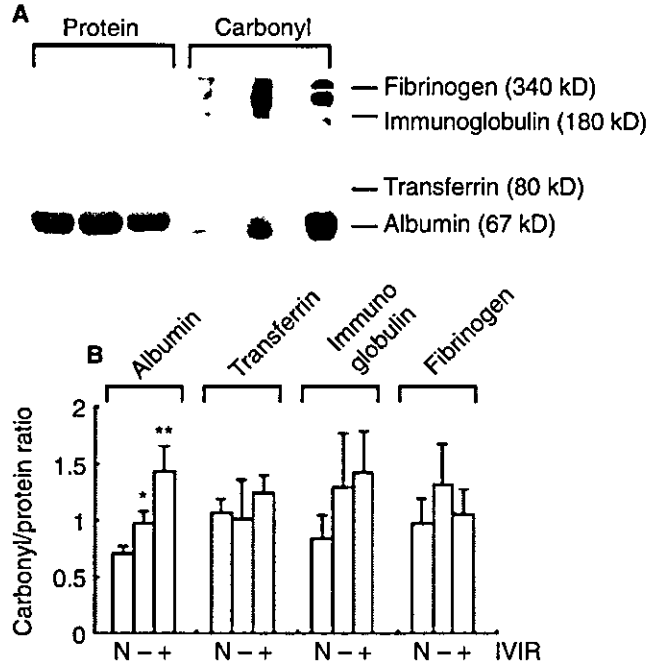

Fig. 3. Carbonyl content of major plasma proteins from normal subjects and HD patients with or without intravenous iron administration (IVIR). Plasma samples from HD patients with or without IVIR were derivatized with DNP and subjected to duplicate SDS-PAGE gels (*A*). Following electrotransfer, one blot was stained with Coomassie brilliant blue G for protein (upper blot) and the second blot was stained for DNP using OxiBlot kit reagents (lower blot). Carbonyl formation of major plasma proteins (albumin, transferrin, immunoglobulin, and fibrinogen) were determined as densitometry ratio of DNP area and protein area, and are reported in densitometry units (*B*). Values are expressed as mean ± SE; $N = 11$ patients per group. $^{*}P < 0.05$ as compared with plasma from patients without IVIR.

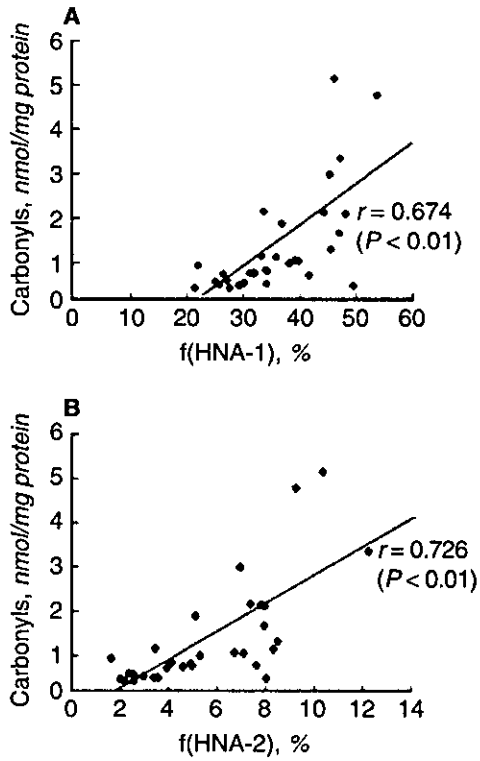

Fig. 4. Relationship between plasma protein carbonyl content and f(HNA-1) (*A*) or f(HNA-2) (*B*). Plasma protein carbonyl contents and f(HNA-1) or f(HNA-2) values from normal subjects and HD patients with or without intravenous iron administration (IVIR) are plotted, and the line shows linear regression of plasma protein carbonyl content and f(HNA-1) or f(HNA-2) values ($N = 33$, $R = 0.674$ and $R = 0.724$, respectively, $P < 0.01$).

Table 4. f(HMA), f(HNA-1), and f(HNA-2) values (%) for reduced and oxidized albumin in sera from healthy subjects and HD patients with or without IVIR

	Healthy subjects ($N = 11$)	Patients without IVIR ($N = 11$)	Patients with IVIR ($N = 11$)
f(HMA)	68.1 ± 2.12	58.5 ± 7.37[a]	50.3 ± 7.25[a]
f(HNA-1)	28.9 ± 1.76	36.0 ± 6.03[a]	41.7 ± 6.27[a,b]
f(HNA-2)	2.99 ± 0.40	5.46 ± 1.50[a]	8.7 ± 2.22[a,b]

Values are expressed as mean ± SE.
[a] $P < 0.05$ as compared with sera from healthy subjects.
[b] $P < 0.05$ as compared with sera from patients without IVIR.

in Figure 1, an HPLC analysis of serum albumin showed a clear separation of HMA, HNA-1, and HNA-2. In HD patients, we found that serum albumin is oxidized, leading to an increase in both f(HNA-1) and f(HNA-2), and that an increase in f(HNA-2) is associated with IVIR. There was no significant difference between the non-IVIR group and IVIR group in several markers that can affect oxidative stress levels, such as dialysis efficiency (Kt/Vurea), uric acid, CRP, and diabetes. These results suggest that HPLC analysis has the potential to provide quantitative as well as qualitative information on the states at oxidation of serum albumin in HD patients. Furthermore, our findings suggest that IVIR is associated with an increase in the oxidized albumin, as evidenced by an increase in f(HNA-2) and plasma protein carbonyl contents.

Cardiovascular diseases continue to be the major cause of both morbidity and mortality for patients on HD therapy. For HD patients, the annual mortality rate caused by cardiovascular disorders is approximately 9%, which is 10- to 20-fold higher than the general population, even when adjusted for age, sex, race, and the presence or absence of diabetes [37]. A potential link between inflammation, hypoalbuminemia, and subsequent cardiovascular risk in HD patients may be through the process of oxidative stress. Although Fe^{2+} is known to be a powerful source of hydroxyl radicals through the Fenton reaction, the majority of HD patients receive IVIR for anemia correction. Because iron loading markedly alters the antioxidant system [38], and because uremic patients have numerous defects in antioxidant defense that are unrelated to iron, iron toxicity could amplify these defects, and IVIR in HD patients may represent a specific condition that enhances iron toxicity through the generation of oxidative stress. This hypothesis is also supported by the