ADP and SS plasma application was reversed. Three of the seven subjects whose platelets were synergistically activated by epinephrine and SS plasma, and three of the nine subjects whose platelets were not activated, were examined. The addition of 1 μM ADP to SS plasma-pretreated platelets elicited full aggregation (Fig. 2b) in the three subjects whose platelets were synergistically activated by epinephrine and SS plasma. RANTES release was also induced (median, 11·4 ng/ml; range: 10·2–22·3 ng/ml; $n = 3$) compared to stimulation with SS plasma alone (median, 1·3 ng/ml; range: 0·2–8·3 ng/ml; $n = 3$). The combination of negative control plasma and ADP did not induce platelet activation (Fig. 2b). Furthermore, platelets from the latter three subjects showed no response under this condition (data not shown). These results suggest that the pretreatment of SS plasma could also cause platelet activation synergistically with the subsequent addition of a low dose of ADP in a subpopulation of subjects whose platelets responded to SS plasma synergistically with epinephrine pretreatment.

Involvement of FcγRIIa in platelet activation induced by SS plasma

As some anti-platelet immunoglobulins, including anti-CD36 murine mAbs, activate platelets in a manner dependent on FcγRIIa [13,15,20–23], we evaluated the role of FcγRIIa in platelet activation via SS plasma by using an anti-FcγRIIa-blocking mAb, IV.3. PRP obtained from subjects whose platelets showed marked activation induced by SS plasma alone was preincubated with 20 μg/ml of IV.3 and then stimulated with SS plasma. IV.3 inhibited platelet activation in all subjects tested. Representative results for three donors are shown in Fig. 3(a). The maximum aggregation for 10 min after stimulation with the plasma and the RANTES level in the cell-free supernatant were significantly reduced by 65·8 ± 18·6% and by 58·3 ± 16·6% (mean ± SE: $n = 3$), respectively, in the presence of 20 μg/ml IV.3 (Fig. 3b,3c). The increase of IV.3 up to 80 μg/ml did not cause further reduction of platelet activation by the plasma (data not shown). In contrast, the control antibody had no inhibitory effects (Fig. 3).

We also studied the effect of IV.3 on platelet activation caused by the synergy between SS plasma and epinephrine. PRP obtained from subjects whose platelets required epinephrine for the full activation in response to SS plasma, was pretreated with epinephrine, and then 20 μg/ml of IV.3 before stimulation with the plasma. The platelet aggregation and RANTES release caused by the synergy between SS plasma and epinephrine were also significantly inhibited by the presence of IV.3 (Fig. 4). In this case, 20 μg/ml of IV.3 reduced platelet aggregation and RANTES release by 92·5 ± 3·7% and 97·3 ± 1·7% (mean ± SE: $n = 3$), respectively (Fig. 4b,4c). The control antibody had no inhibitory effects on the platelet aggregation (Fig. 4b). The reduction of RANTES release by the control antibody [15·2 ± 3·2% (mean ± SE); $n = 3$] was very slight compared to that by IV.3 (Fig. 4c). These results suggest that FcγRIIa is involved in the platelet activation by SS plasma alone or in synergy with epinephrine.

Responsiveness of platelets and the binding of SS serum to platelets

To investigate interindividual variations in the platelet response to SS plasma, we examined the relationship between the responsiveness of platelets and the platelet surface level of CD36 or the binding of SS serum to platelets. Subjects could be consequently divided into groups 1, 2, or 3 on the basis of the heterogeneity of the responsiveness of the platelets to the plasma. In group 1, the platelets were activated by SS plasma alone; in group 2, the platelets were only activated by synergy between SS plasma and epinephrine; and in group 3, the platelets failed to respond to the plasma under the conditions tested. The MFI of the binding of the anti-CD36 mAbs to platelets, and the MFI of the binding of SS serum to platelets, as determined by flow cytometry, were then compared among these three groups. The MFI of anti-CD36 mAbs binding to platelets in group 1 was significantly higher than those in groups 2 and 3 (Fig. 5a). There was no significant difference between groups 2 and 3. The MFI of the binding of SS serum to the platelets in group 1 was significantly higher than that in group 3 (Fig. 5b). There was no significant difference between group 2 and the other two groups (Fig. 5b). The MFI of the binding of SS serum correlated with the MFI of anti-CD36 mAb binding ($r^2 = 0·794$, $P < 0·01$). These results suggest that the surface levels of CD36 and the degree of SS serum binding are associated with the differences in platelet response to SS plasma observed between groups 1 and 3.

The responsiveness of platelets and FcγRIIa: expression and polymorphism

We then investigated the involvement of FcγRIIa in the heterogeneity of platelet responsiveness to SS plasma. In order to do so, the surface level and polymorphism at amino acid 131 of FcγRIIa were examined. The MFI of the IV.3 binding to platelets, as determined by flow cytometry, was compared among these three groups. The MFI of the IV.3 binding to platelets in group 1 was significantly higher than that in group 3 (Fig. 5c). There was no significant difference between group 2 and the other two groups. Among the 16 subjects with CD36-positive platelets available for the study of the polymorphism, nine were homozygous His, six were heterozygous Arg/His, and one was homozygous Arg (Table 2). The platelets of subjects 1, 2 and 3 (from group 1), 5, 6 and 7 (from group 2), and 11, 12 and 13 (from group 3), were His/His in the phenotype of FcγRIIa, but exhibited a

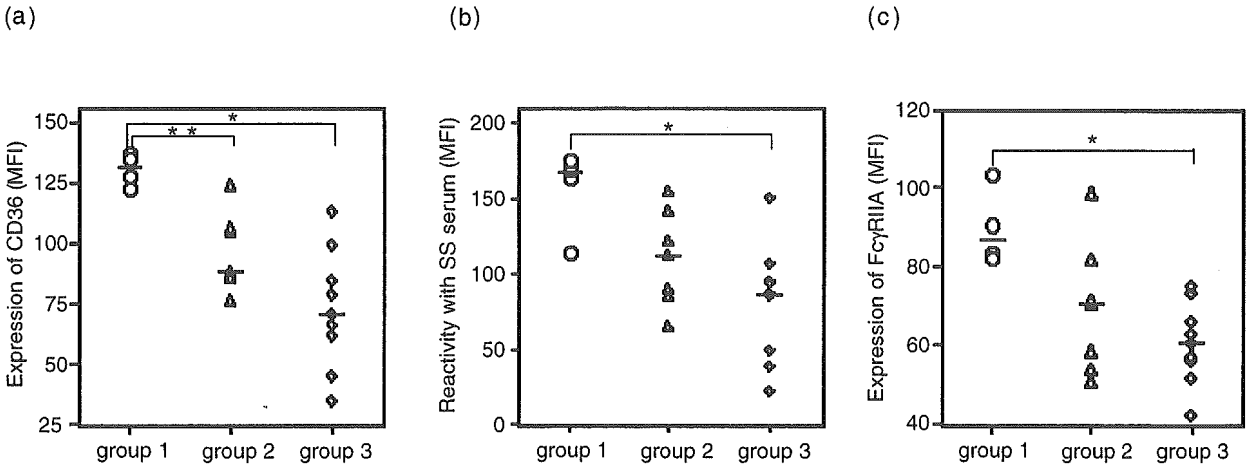

Fig. 5 Heterogeneity of platelet responsiveness to SS plasma, surface levels of CD36 and FcγRIIa, and binding of SS serum to platelets. Subjects were divided into groups 1 (n = 4), 2 (n = 7), and 3 (n = 9) on the basis of heterogeneity of responsiveness of platelets to SS plasma, as described in the Results. The mean fluorescence intensity (MFI) of the binding of the anti-CD36 mAb (a), SS serum with platelets (b) and IV.3 (c) determined by flow cytometry were compared among these three groups. (a) $^{*}P < 0{\cdot}016$ group 1 vs. group 3. $^{**}P < 0{\cdot}016$ group 1 vs. group 2. (b) $^{*}P < 0{\cdot}016$ group 1 vs. group 3. (c) $^{*}P < 0{\cdot}016$ group 1 vs. group 3.

variable responsiveness to the plasma. Similarly, the platelets of subjects 4 (from group 1), 8 and 9 (from group 2), and 14, 15 and 16 (from group 3) were Arg/His, but again differed in the responsiveness to the plasma. The platelets of subject 10 (from group 2) were Arg/Arg. Therefore, there was no correlation between the responsiveness of platelets to the plasma and the polymorphisms of FcγRIIa. These results suggest that the surface expression level of FcγRIIa participated in the different responsiveness of platelets to SS plasma between groups 1 and 3. However, the polymorphisms of FcγRIIa were not involved in the heterogeneity of the responsiveness.

Platelet activation induced by anti-CD36-containing sera derived from patients who developed refractoriness to platelet transfusion or random healthy donors

To verify whether the platelet activation observed in our experiment was exclusively limited to SS plasma, we examined the platelet-activating ability of other anti-CD36-containing sera derived from patients who developed refractoriness to platelet transfusion (n = 1) or random healthy donors (n = 12). PRP obtained from subjects in group 1 was incubated with these anti-CD36-containing sera. In addition to the SS serum, one anti-CD36-containing serum (serum sample no. 1) from a random healthy donor caused remarkable platelet aggregation (Table 3). SS serum and serum sample no. 1 did not induce platelet activation in CD36-negative platelets and consequently showed a specific response to CD36. The two platelet-activating sera contained a relatively higher titre of anti-CD36 than the other sera (Table 3). The limited amount of serum available prevented us from testing whether a similar heterogeneity in platelet response may exist in another platelet-activating anti-CD36-containing serum. These results suggest that the platelet activation induced by the anti-CD36-containing serum was not specific to the anti-CD36-containing serum sample derived from donor SS.

Table 3 Aggregation of platelets induced by anti-CD36-containing sera

	CD36 [+]				
Serum sample[c]	Subject no. 2[d]	Subject no. 3[d]	Subject no. 4[d]	CD36[–]	Titre
SS serum	+[a]	+	+	–	×512
No. 1	+	+	+	–	×512
No. 2	–[b]	–	–	NT	×2
No. 3	–	–	–	NT	×2
No. 4	–	–	–	NT	×4
No. 5	–	–	–	NT	×4
No. 6	–	–	–	NT	×16
No. 7	–	–	–	NT	×16
No. 8	NT	–	–	NT	×32
No. 9	–	–	–	NT	×128
No. 10	–	–	–	NT	×128
No. 11	NT	–	–	NT	×128
No. 12	NT	–	–	NT	×128
No. 13	–	–	–	NT	×32

[a]Platelets showed aggregation.
[b]Platelets showed no response.
[c]SS serum and serum samples 1–12 are derived from random healthy donors. Serum sample 13 is derived from patients who developed refractoriness to platelet transfusion.
[d]Subjects 2, 3 and 4 correspond to subjects 2, 3 and 4 in Table 2.
NT, not tested.

Discussion

We investigated the *in vitro* responses of platelets derived from healthy subjects to anti-CD36 that had been detected in FFP implicated in the development of NHTRs and thrombocytopenia. We used anti-CD36-containing plasma (i.e. SS plasma) without purification and PRP as targets. We assumed that the use of PRP in combination with plasma mimics allogeneic transfusion more closely than the use of washed platelets with purified antibody. Considerable heterogeneity in platelet responsiveness to SS plasma was observed and was divided into three groups. The observed response was caused by activation of the platelet signalling pathway, and not by passive agglutination, because PGE_1 inhibited the platelet activation induced by this plasma. Furthermore, FcγRIIa was required for the platelet activation induced by both SS plasma (i.e. group 1) and by the synergy of this plasma and epinephrine priming (i.e. group 2). The heterogeneity of the platelet response, and the dependence of the response on FcγRIIa, agree with findings for other anti-platelet immunoglobulins [12–14,16–18,20–22].

To explain the heterogeneity of platelet responsiveness to SS plasma, we analysed the surface expression levels of CD36 and FcγRIIa on platelets and the binding of SS serum to platelets among the three groups. The flow cytometric analysis revealed that the surface expression levels of CD36 and FcγRIIa on the platelets, and the binding of the SS serum to platelets in group 1, were significantly higher than those in group 3. Thus, the surface expression levels of CD36 and FcγRIIa, as well as the degree of binding to the SS serum, were thought to be associated with the profound difference in platelet responsiveness to SS plasma, at least between groups 1 and 3. In other words, higher surface levels of CD36 and FcγRIIa may be necessary for the platelet activation induced by SS plasma alone. Further analysis of a larger number of specimens is needed to confirm these findings. On the other hand, the differences in platelet responsiveness between group 2 and the other two groups could not be explained by the surface levels of CD36 and FcγRIIa, or by the binding of SS serum, although the levels of these molecules seen in group 2 were of intermediate value, compared to the levels of the other groups. The determinants of the differences between group 2 and the other two groups are not clear, at present. Interindividual differences in the sensitivity of platelets to epinephrine have been well documented. However, it is unlikely that the diverse sensitivity to epinephrine contributes to the differences seen between groups 2 and 3 because titrations were performed to find the concentration of epinephrine that elicited an aggregation of less than 20%, and significant differences in those concentrations were not observed between groups 2 and 3.

With respect to FcγRIIa polymorphisms, murine IgG1 mAbs have been shown to have a higher affinity for the Arg131 phenotype than for the His131 phenotype. The degree of affinity was correlated with the intensity of the platelet response to murine platelet-activating mAbs [13,20,24–26]. In human IgG, the IgG2 subclass depends primarily on the presence of FcγRIIa polymorphisms with opposite affinity; in other words, IgG2 binds preferentially to the His131 phenotype [24]. However, FcγRIIa polymorphisms have also been shown to influence the affinity of human IgG1 subclass Abs in the case of HIT sera: platelets that are homozygous for the His131 polymorphism of FcγRIIa exhibit the greatest reactivity to HIT-IgG1 [18]. Because the subclass of anti-CD36 detected in the plasma was IgG1 dominant, we hypothesized that the same relationship might apply in the present study. However, we did not observe any correlation between the FcγRIIa polymorphisms and platelet responsiveness to SS plasma in the limited number of subjects examined in the present study.

In addition to the FcγRIIa-dependent pathway, FcγRIIa-independent mechanisms are also known to lead to anti-platelet immunoglobulin-induced platelet activation [23]. The latter mechanisms include the following pathways [23]: platelet-bound antibodies can directly induce platelet activation by binding to their target antigen; and platelet activation can occur indirectly through antibody-mediated complement activation. Thus, group 1 may require both FcγRIIa-dependent and -independent pathways. Additionally, the presence of unknown factors in SS plasma might be associated with the heterogeneity observed in this study. The details of the mechanism underlying platelet activation induced by anti-CD36 alone and in synergy remain to be determined.

In this study, platelet activation was not specific to the anti-CD36 sample derived from donor SS. In addition to the SS serum, one out of 13 anti-CD36-containing serum samples also caused platelet aggregation. The two platelet-activating sera contained a relatively higher titre of anti-CD36 than the other sera. This is thought to be the reason why 12 out of 14 anti-CD36-containing serum samples had no effect on the platelets. Although the possibility that these 12 sera might induce platelet activation under different conditions, such as a higher serum volume than that used in the present study, cannot be ruled out, the limited amount of available sera prevented further analysis.

The possible clinical significance of platelet activation by anti-platelet immunoglobulin has been proposed in the pathology of anti-platelet immunoglobulin-related clinical symptoms [9–12,33]. Although the clinical significance of the platelet activation induced by anti-CD36 is uncertain at present, the presence of a subpopulation of subjects whose platelets were activated by anti-CD36 derived from two independent sources of sera implies that the passive transfusion of anti-CD36 may be a risk factor for the occurrence of NHTRs in this subpopulation. The synergistic effect of epinephrine priming and SS plasma in a subpopulation

of subjects also indicates that some priming events of the platelets *in vivo* may confer responsiveness to anti-CD36 in this subpopulation, thereby resulting in the manifestation of platelet activation-triggered NHTRs.

In conclusion, platelets derived from normal healthy subjects showed considerable heterogeneity in platelet responsiveness to the anti-CD36 that are implicated in NHTRs and thrombocytopenia. Our findings raise the possibility that the heterogeneous response to anti-CD36 may influence the occurrence of adverse effects after the transfusion of anti-CD36-containing blood components.

Acknowledgements

The authors wish to acknowledge the following individuals for providing the sera containing anti-CD36: Fumiaki Nakajima (Kanagawa Red Cross Blood Center, Yokohama, Japan); Shoji Morita (Japanese Red Cross Saitama Blood Center, Hidaka, Japan); Yoshiko Teraki (Chiba Red Cross Blood Center, Chiba, Japan); Hiroyuki Sato (Fukuoka Red Cross Blood Center, Fukuoka, Japan); and Yasuo Fukumori (Osaka Red Cross Blood Center, Osaka, Japan).

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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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Hiroshi Azuma, Hisami Ikeda

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．

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.

Keywords

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

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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論文受付 2004年11月９日　論文受理 2004年12月21日

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

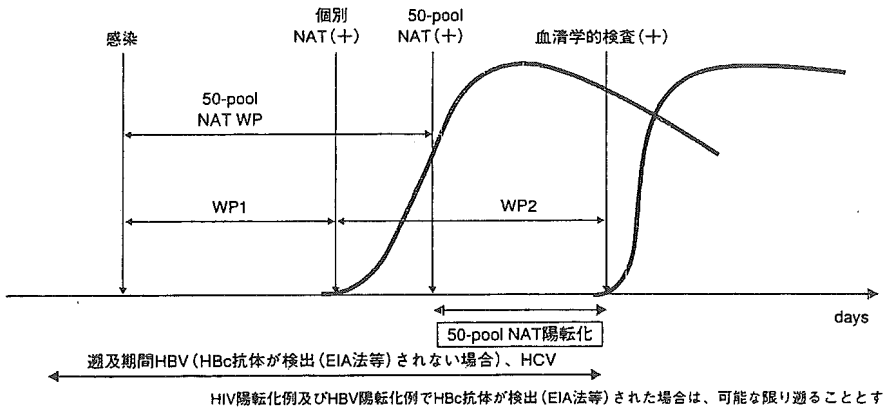
表1

	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陰性時期の遡及機間は各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）



①遡及期間は50-pool NAT陽転化時の起点として、HBV（HBc抗体が検出されない場合）は125日、HCVは192日以内とする。また、HIVは可能な限り遡ることとする。なお、HBV50-pool NAT陽転時でHBc抗体（EIA法等）が検出された場合は、可能な限り過去に遡り、保管検体の個別NATが陰性と判定されるまで遡及する。
［50-pool NAT陽転化例（急性感染）感染時期は、血清学的検査のウインドウ期間内（WP1＋WP2）に存在する］

②上記の遡及期間内の範囲で直近前回及びその過去92日（HBV：HBc抗体が検出されない場合）、50日（HCV）または58日（HIV）以内のすべての輸血用血液、原料血漿を遡及調査の対象とする。
［直近前回が50-pool NAT陰性であれば、感染時期は50-pool NAT WPの期間内に存在する］

＊この対応により陽転化例の発生後、迅速に一括して回収・遡及調査が可能になる。

（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）

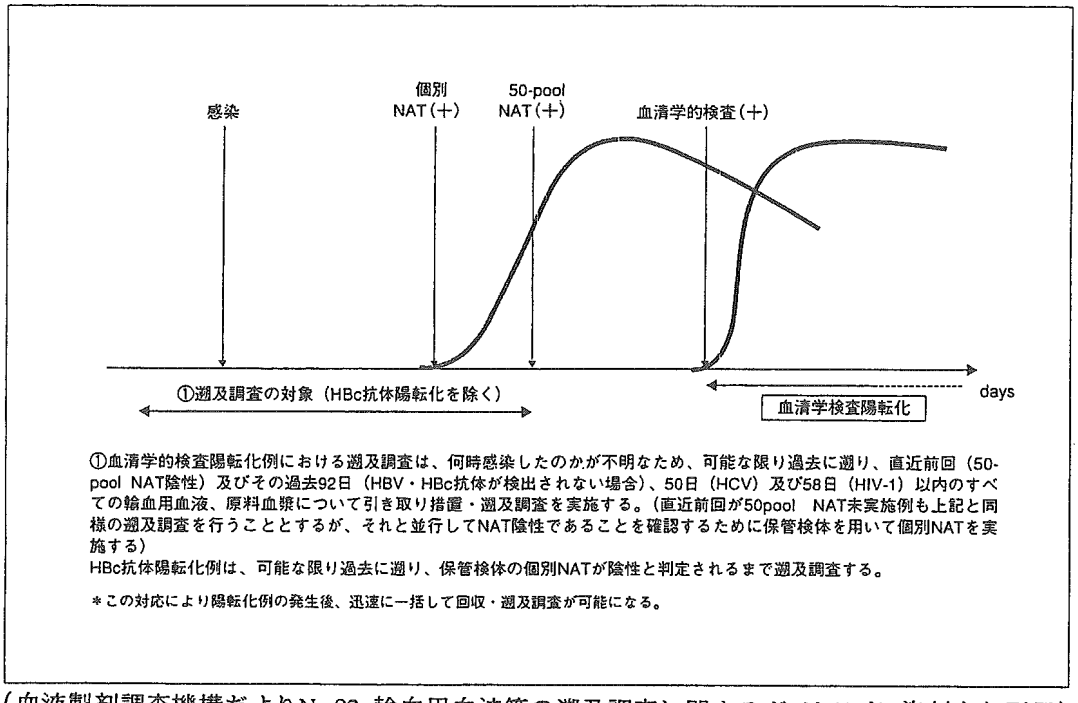
図1．50-pool NAT陽転化例の溯及期間

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$1x10^{6-7}$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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0090-9556/04/3210-1069–1074$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 32, No. 10
513/1173611
Printed in U.S.A.

DMD 32:1069–1074, 2004

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

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Received May 4, 2004; accepted June 25, 2004

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*.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.104.000513.

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.

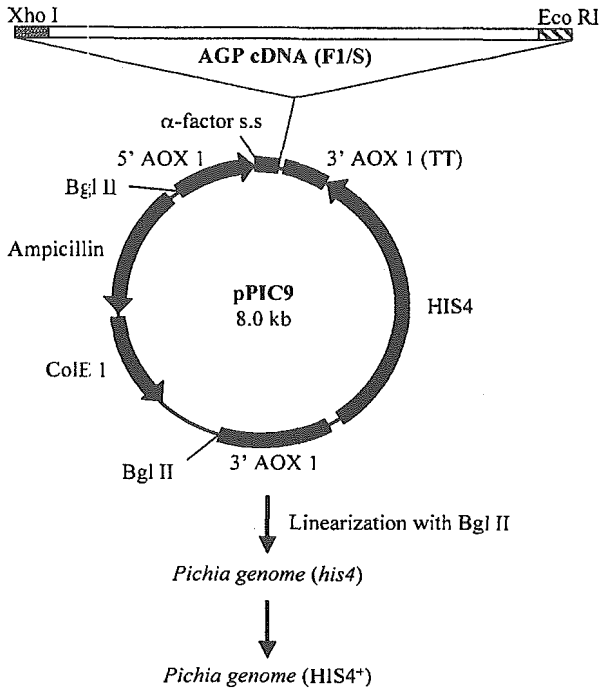

FIG. 1. Expression construct for rhAGP in *Pichia pastoris* (GS115).

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.

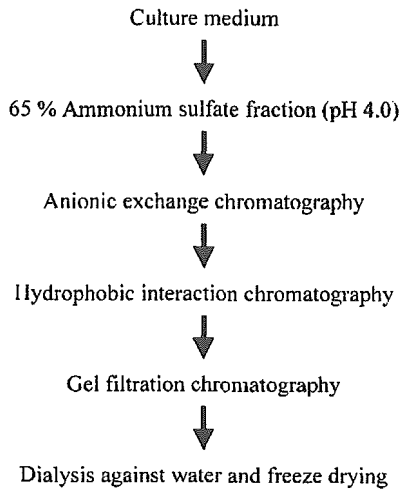

FIG. 2. Flow chart for purification of rhAGP.

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

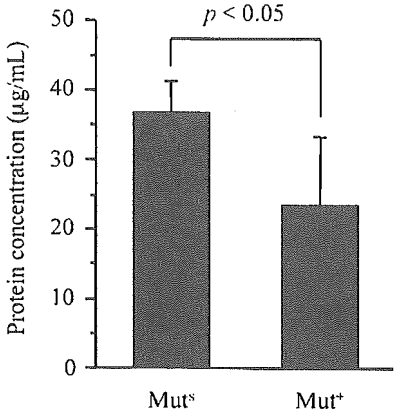

FIG. 3. Screening of secreted colonies (Mut^S and Mut$^+$).

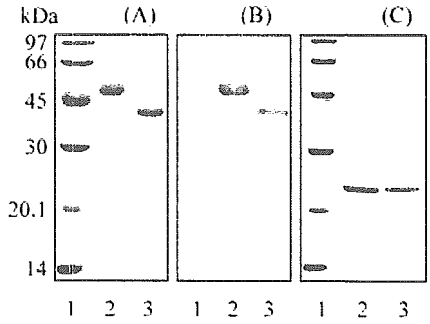

FIG. 4. SDS-PAGE and Western blot analysis of hAGP (lane 2) and rhAGP (lane 3). A, SDS-PAGE of hAGP and rhAGP. B, Western blot analysis of hAGP and rhAGP using hAGP polyclonal antibody. C, SDS-PAGE of hAGP and rhAGP treated with GPF. Lane 1 represents a molecular weight marker.

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.

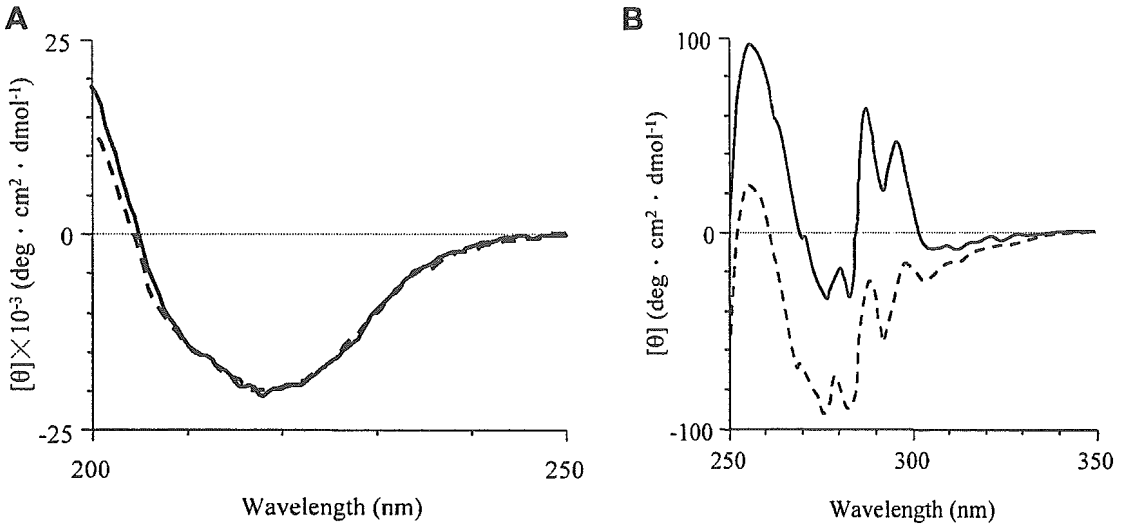

FIG. 5. Far- (A) and near- (B) UV CD spectra of hAGP (solid line) and rhAGP (dashed line).

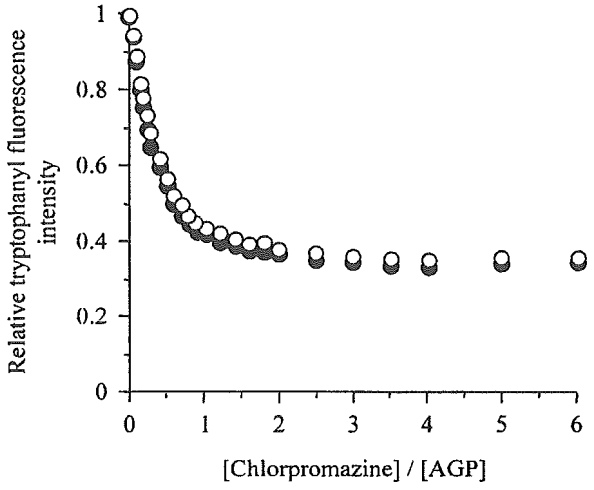

FIG. 6. Quenching of tryptophanyl fluorescence of AGP by chlorpromazine. Symbols represent hAGP (filled circles) and rhAGP (open circles), respectively.

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

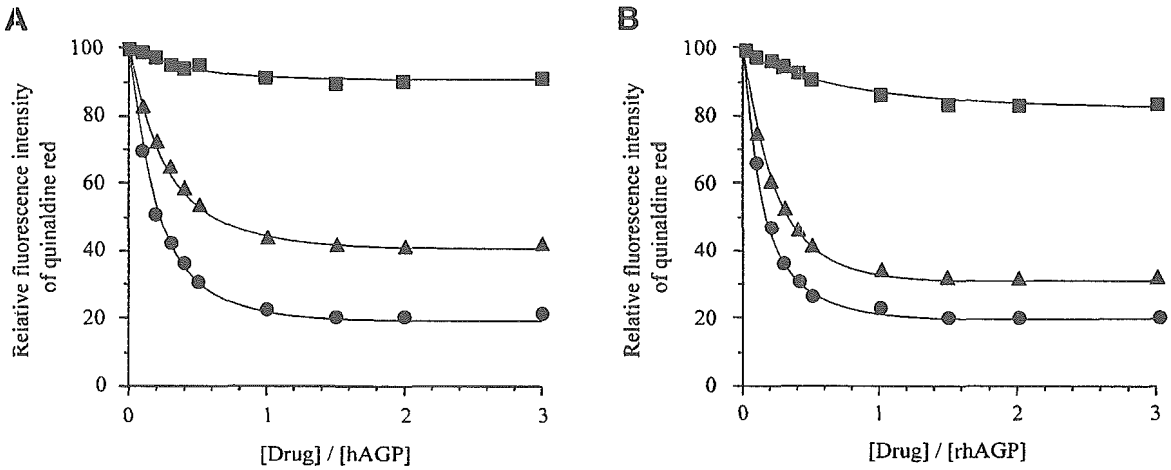

FIG. 7. Displacement of quinaldine red by AGP-binding drug. The experiments were performed at a quinaldine red concentration of 10 μM, and hAGP (A) and rhAGP (B) concentration of 10 μM. Drugs used in this experiment were as follows: progesterone (squares), warfarin (triangles), and chlorpromazine (circles).

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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Kidney International, Vol. 66 (2004), pp. 841–848

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.

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

Received for publication August 21, 2003
and in revised form December 29, 2003, and February 2, 2004
Accepted for publication March 10, 2004

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.