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.

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EFFECTS OF HEMOGLOBIN VESICLES ON RESTING AND AGONIST-STIMULATED HUMAN PLATELETS IN VITRO

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ABSTRACT

Hemoglobin vesicles (HbV) are artificial oxygen carriers that encapsulate a concentrated hemoglobin (Hb) solution with a phospholipid bilayer membrane. The oxygen transporting ability of HbV in vivo has been demonstrated by the transfusion of HbV into hemorrhagic shock rodent models. However, the compatibility of HbV with human blood cells must be evaluated. Preincubation of platelets with concentrations of 20% or 40% HbV had no effect on the binding of PAC-1, a monoclonal antibody that detects activation-dependent conformational changes in $\alpha_{IIb}\beta_3$ on platelets, or the surface expression of CD62P in whole blood. ADP-induced increases in PAC-1 binding were significantly enhanced by exposing the platelets to concentrations of either 20% or 40% HbV, whereas the ADP-induced increases in CD62P expression were not affected by HbV treatment at either concentration. Preincubation of platelet-rich plasma (PRP) with HbV minimally reduced the spontaneous release of TXB_2 and RANTES, but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with ADP. Similarly, preincubation of PRP with HbV minimally

reduced the spontaneous release of RANTES but did not significantly affect the formation of TXB_2 or the release of RANTES and β-TG in platelets stimulated with collagen, although collagen-induced serotonin release tended to decrease with HbV pretreatment. These data suggest that the exposure of human platelets to high concentrations of HbV (up to 40%) in vitro did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances triggered by platelet agonists, although one of the earliest events in ADP-induced platelet activation was slightly potentiated by HbV pretreatment at the doses tested. Taken together, these results imply that HbV, at concentrations of up to 40%, do not have any aberrant interactions with either unstimulated or agonist-induced platelets.

INTRODUCTION

Vigorous efforts have been made to develop hemoglobin (Hb)-based oxygen carriers (HBOCs) for use as red blood cell substitutes [1], and some of these carriers are now in the final stages of clinical trials [2-4]. HBOCs offer several potential benefits for red

blood cell transfusion applications, including the absence of blood-type antigens and infectious viruses and the ability to be stably stored for long time periods [5]. HBOCs can be categorized into two types: acellular modified Hb molecules and cellular liposome-encapsulated Hb, or Hb vesicles (HbV) [6]. Acellular modified Hb molecules are composed of intramolecularly cross-linked Hb, recombinant cross-linked Hb, polymerized Hb, or intramolecularly polymer-conjugated Hb. An acellular polymerized bovine Hb has already been used in clinical practice in South Africa.

Cellular HbV have a phospholipid vesicle structure and contain concentrated Hb molecules, similar to actual red blood cells [7-11]. Although HbV have not been clinically tested, the oxygen transporting abilities of HbV have been shown to be sufficient using a 40% exchange transfusion with HbV suspended in saline [8] and a 90% exchange transfusion with HbV in the presence of albumin as a plasma expander in rats [7]. Surface modification of HbV with poly(ethyleneglycol)-phospatidylethanolamine reduced the viscosity by suppressing inter-vesicular aggregation, allowing prompt blood circulation in vivo [9]. A sufficient O_2 transporting

ability, comparable with that of blood, was also established in another model [11], and the prompt metabolism of HbV in the reticulo-endothelial system has been demonstrated [10].

The biocompatibility of HbV is an important factor for the clinical use of these materials. The administration of HbV could lead to interactions with blood cells, including platelets. Circulating platelets bind to the subendothelial matrix of injured vessels and subsequently become activated, resulting in the release or the expression of components in their intracellular granules and the formation of metabolic products. These products include prothrombotic substances (e.g., adenine nucleotides, thromboxane A_2 [TXA_2], serotonin, and CD62P) [12] and an array of potent proinflammatory chemokines (e.g., RANTES, MIP-1) [13]. Prothrombotic substances function as agonists for the recruitment of additional platelets into the evolving thrombus. Chemokines released from the activated platelets trigger the recruitment of leukocytes into the evolving thrombus and play a large role in the initiation and perpetuation of inflammatory responses. Platelet activation is apparently necessary to

prevent bleeding in vivo; however, nonphysiological activation leads to pathological thrombosis and the modulation of inflammatory responses. With this in mind, the biocompatibility of HbV and human platelets was evaluated by examining the effect of HbV on CD62P expression and the binding of activation–dependent $\alpha_{IIb}\beta_3$ antibody PAC-1 to platelets in the presence or absence of agonists in vitro; these two markers are the most frequently used markers of platelet activation. We also studied the effects of HbV on the secretion of other substances (i.e., serotonin, RANTES, and β-thromboglobulin [β-TG]) and the formation of thromboxane B_2 (TXB_2), a metabolite of TXA_2.

MATERIALS AND METHODS

HbV

HbV suspended in phosphate buffered saline were prepared as previously described [14]. The encapsulated carbonylhemoglobin contained pyridoxal 5′-phosphate (PLP) at a molar ratio of [Hb]/[PLP] = 1/2.5 as an allosteric effector and 5 mM of DL-homocysteine. The lipid bilayer was composed of 1,2-dipalmitoyl-sn-glycero-3-

phosphatidylcholine, cholesterol, 1,5-dipalmitoyl-L-glutamate-*N*-succinic acid, and polyethyleneglycol-1, 2-distearyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly (ethylene glycol) (5,000)] at a molar ratio of 5:5:1:0.033. The Hb concentration of the HbV dispersion was adjusted to 10 g/dl. The HbV particle size was nearly 240±60 nm in diameter.

Determination of CD62P and PAC-1 expression by flow cytometry

The expression of CD62P and PAC-1 on platelets was measured as described previously, with slight modifications [15, 16]. Citrated whole blood was obtained from unselected healthy subjects. Whole blood (520 μl) was incubated with 480 μl of HbV or empty liposomes (at concentrations of 0%, 20%, or 40%) at 37°C for 60 minutes. After incubation, the reaction mixture was diluted to 1/5.4 with Hepes-Tyrode's buffer (KCl, 2 mM; NaCl, 127 mM; NaH_2PO_4, 0.5 mM; glucose, 5.6 mM; $NaHCO_3$,12 mM; HEPES, 5 mM; 0.35% BSA; pH7.3). Eighteen microliters of the diluted reaction mixture was added to 18 μl of a cocktail of FITC-conjugated PAC-1, PE-conjugated anti-CD62P and PerCP-conjugated anti-CD42a. FITC-conjugated anti-mouse IgM, PE-conjugated anti-

A two-way repeated measures ANOVA with Bonferroni correction was used for multiple comparisons of mediator levels and surface marker levels among different concentrations of HbV. A p value < 0.05 was considered to indicate a significant difference.

RESULTS

Effect of HbV on the binding of PAC-1 and the expression of CD62P on resting and ADP-stimulated platelets in vitro in whole blood.

First, the effect of HbV on the binding of PAC-1 to platelets and the surface expression of CD62P on platelets with or without ADP stimulation was examined in a whole blood environment in vitro. Without ADP stimulation, PAC-1 binding to platelets was discernible. Preincubation of whole blood with 20% or 40% HbV alone did not cause a significant difference in PAC-1 binding to the platelets. Stimulation of platelets with varying concentrations of ADP caused a gradual increase in the percentage of PAC-1 positive cells (Fig. 1A). Preincubation of whole blood with 20% or 40% HbV resulted in a slight, but significant, enhancement in the percentage of PAC-1 positive cells,

compared to the results of comparable experiments without HbV, at ADP concentrations ranging from 0.05 μM to 5 μM (Fig. 1A).

Unstimulated platelets showed only a slight expression of CD62P, regardless of HbV treatment (Fig. 1B). The treatment of platelets with varying concentrations of ADP also led to gradual increases in the percentage of CD62P-positive cells, but preincubation of whole blood with 20% or 40% HbV did not affect the ADP-induced increase in the percentage of CD62P-positive cells (Fig. 1B).

Effect of HbV on secretion of platelet-derived mediators in resting and ADP-stimulated platelets in vitro.

Next, the effect of HbV on the release of mediators from platelets stimulated with or without a submaximal dose of ADP, a weak platelet agonist, was examined. Without ADP stimulation, a slight, but significant, reduction in the spontaneous release of TXB_2 from platelets pretreated with 40% HbV was observed (Fig. 2A). Similarly, the levels of spontaneous release of RANTES from platelets pretreated with both 20% and 40% HbV

Activated platelets secrete a number of prothrombotic substances, like TXA_2, serotonin, and CD62P that act synergistically to form thrombi. TXA_2 is synthesized via the cyclooxygenase-mediated arachidonic metabolic pathway [20] and is a potent platelet agonist that induces a rapid positive feedback loop, thereby amplifying the activation signals and enabling robust platelet recruitment at the site of vascular injury [21]. Serotonin is a bioactive amine that localizes in dense granules of resting platelets and is secreted upon platelet activation. Serotonin also has a prothrombotic effect on platelets [12]. Interactions between platelets via CD62P stabilize the initial $\alpha_{IIb}\beta_3$ –fibrinogen interactions, thereby promoting the formation of large, stable platelet aggregates [22]. In addition, CD62P is the major surface receptor for neutrophils and monocytes on activated platelets, mediating leukocyte adhesion. Thus, platelet CD62P is involved in the recruitment of both platelets and leukocytes into an evolving thrombus [22-25].

Recent studies have extended platelet function to include the modulation of local inflammatory events through the release of chemokines and cytokines [13]. RANTES and β-TG [13] are stored in α-granules in platelets and are released from platelets on

activation. RANTES has diverse inflammatory effects, such as histamine release from basophils and the exocytosis of eosinophil cationic protein. Furthermore, RANTES is a powerful chemoattractant for T cells, basophils, and eosinophils [13, 26]. β-TG, a platelet-derived CXC chemokine, is also released into the blood at micromolar concentrations and plays an important role in the recruitment of neutrophils to sites of tissue injury [27]. Consequently, the aberrant release of serotonin, TXA_2, RANTES, or β-TG in response to the inappropriate activation of platelets could result in pathological thrombosis, inflammatory reactions, or allergic responses.

The present study demonstrated that the exposure of blood samples to HbV at concentrations of up to 40% did not cause platelet activation, as measured by various markers, although the levels of RANTES and TXB_2 were significantly, but marginally, reduced. In terms of the effect of HbV on platelet activation triggered by submaximal concentrations of agonists, the enhancement of PAC-1 binding to platelets, one of the earliest markers of activation, was observed after the exposure of blood samples to HbV at concentrations of up to 40%, suggesting that HbV might have an enhancing effect on

agonist-induced platelet aggregation. Other than the slight enhancing effect of HbV on PAC-1 binding in the presence of agonist stimulation, however, none of the other parameters were significantly affected. Rather, the levels of TXB_2, RANTES, and β-TG tended to be reduced by ADP stimulation, while the level of serotonin tended to be reduced by collagen stimulation. Thus, the lack of coordinated potentiation in the levels of prothrombotic substances (i.e., TXB_2 and serotonin) and stabilizing molecules involved in the initial $\alpha_{IIb}\beta_3$ – fibrinogen interactions (i.e., CD62P) in response to the presence of an agonist suggests that the enhancing effect of HbV on platelet reactivity to agonists, if present, is not likely to lead to the deleterious formation of thrombi. In addition, the absence of adverse effects on the secretion of RANTES and β-TG suggest that HbV is unlikely to trigger the initiation and/or aberrant perpetuation of inflammatory and allergic reactions.

The present results are of value for estimating the biocompatibility of HbV and human platelets. Further research is warranted to investigate whether the administration of HbV has any effect on platelet activation and platelet functions in vivo.

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LEGENDS

FIGURE 1: Effect of HbV on platelet surface activation markers. (A) PAC-1 binding to platelets and (B) CD62P expression on platelets. Whole blood was incubated with HbV at concentrations of 0% (square), 20% (triangle), or 40% (circle). Whole blood was then stimulated with or without various concentrations of ADP, as described in the Materials

and Methods section. Values are the means ± SE of 4 experiments. *$p < 0.05$, **$p < 0.01$, compared with control (0% HbV)

FIGURE 2: Effect of HbV on ADP-induced platelet mediator release.

ADP-induced release of (A) TXB_2, (B) RANTES, and (C) β-TG from human platelets. PRP was incubated with concentrations of 0%, 20%, or 40% HbV and then stimulated with (hatched columns) or without (open columns) ADP, as described in the Materials and Methods section. Values are the means ± SE of 5 (A) and 6 (B, C) experiments using blood from different donors. *$p < 0.05$, compared with control (0% HbV)

FIGURE 3: Effect of HbV on collagen-induced platelet mediator release.

Collagen-induced release of (A) serotonin, (B) TXB_2, (C) RANTES, and (D) β-TG from platelets. PRP was incubated with concentrations of 0%, 20%, or 40% HbV and then stimulated with (hatched columns) or without (open columns) collagen, as described in the Materials and Methods section. Values are the means ± SE of 5 experiments using blood from different donors. *$p < 0.05$, compared with control (0% HbV)

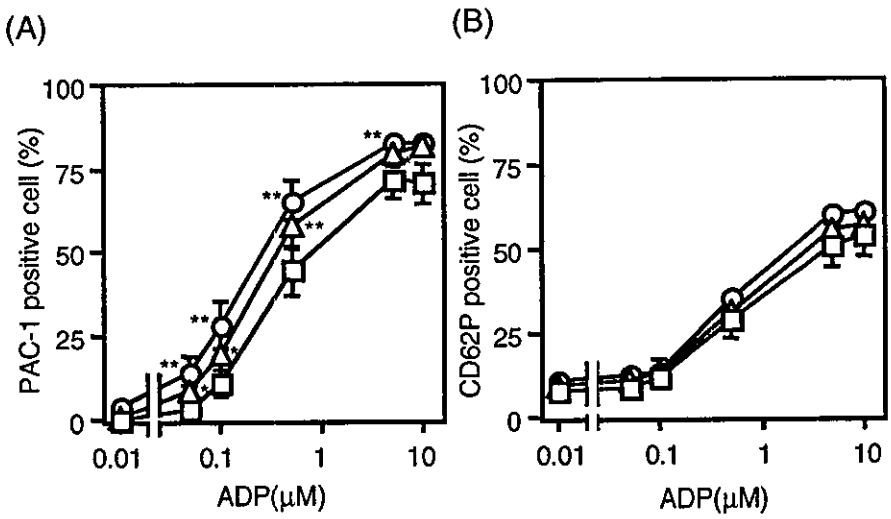

Fig. 1

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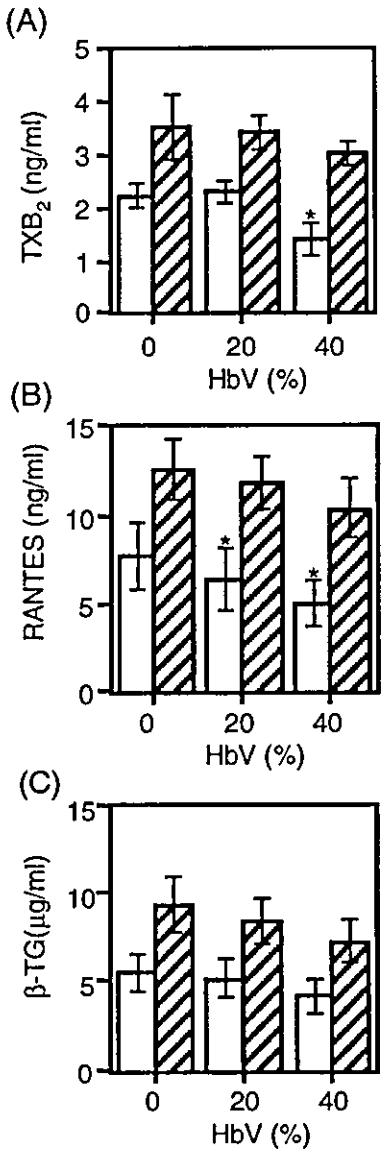

Fig. 2

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