The Origin of ABH Antigens on Human Platelets

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ABH antigens are present on platelets from individuals of the corresponding red cell phenotype, but the extent to which these antigens are intrinsic or adsorbed remains undefined. To evaluate platelets for intrinsic H substance, an IgM mouse monoclonal antibody against type 2H chain (the intrinsic H structure found on erythrocytes) was labeled with 125I and incubated with platelets from donors of different ABO type. The antibody showed dose-response saturation curves, and binding to platelets paralleled that of the red cell ABO type, with 0 > B > A1 > A1B > O, cells, giving a single factor variance F of 190 (P < .0005). Passive adsorption of A antigens by platelets has been previously reported. To verify this phenomenon for A and B antigens and to quantitate the elution of A and B antigens from platelets, the following assay system was used. Platelets from group A, and B donors were incubated in plasma from group O donors, and platelets from group O donors were incubated in plasma from different ABO.

The circulating human platelet readily acquires both ABH and HLA antigens by passive adsorption from the surrounding plasma.1 4 The soluble ABH antigens involved in this phenomenon presumably reside on glycolipid molecules possessing the type 1H chain, while ABO antigens intrinsic to red cell membranes are composed of type 2H chains.5 These two precursor molecules of the A and B antigens differ in the linkage of the subterminal galactose to N-acetyl-galactosamine, with type 1 chain showing β, 1–3 linkage and type 2 chain involving β, 1–4 linkage.5 7 Although platelets are known to possess the ABH antigens corresponding to the ABO red cell type of the individual,1 4 the extent to which these platelet antigens are intrinsic (type 2H chain) and adsorbed (type 1H chain) has not been delineated.

We have used radioimmunoassays, sensitive over a wide range of antigen and antibody concentrations, to examine the human platelet for intrinsic and adsorbed ABH antigens. Evaluation of intrinsic antigens was performed using a direct assay with radiolabeled mouse monoclonal anti-2H antibody.8

The adsorption of A antigen onto group O platelets and the subsequent elution of adsorbed antigen over a 60-minute time course has been studied by Kelton et al.,2 who used a mixed erythrocyte-platelet agglutination test. This assay is subject to a number of errors because of nonspecific cell clumping and the difficulty of visual interpretation. In addition to confirming the rate of A antigen adsorption onto group O platelets with our assay, we wished to verify that the same process occurs with B antigen. Furthermore, we have studied the elution of both A and B antigen from these "coated" platelets and from genetic group A and B platelets over a 24-hour time period.

A two-stage assay with radiolabeled mouse monoclonal anti-human IgG was used to evaluate binding of anti-A and anti-B to platelets incubated in plasma from various ABO, Lewis, and presumed secretor-type donors. The presence and variability of intrinsic (2H) and extrinsic (1H) antigens on platelets may affect the recovery and survival of transfused platelets when donor-recipient mismatches involve the ABO system.

MATERIALS AND METHODS

Collection of Cells and Serums

Whole blood, cells, and serums were collected from staff members in the Duke University Medical Center Laboratories. Fresh and previously frozen human serums were used. The human group O serums containing IgG anti-A or anti-B were supplied by the Carolinas Region American Red Cross Blood Service. An IgG fraction was prepared from each serum by precipitation with caprylic acid and ammonium sulfate.9 Antiserums were screened before use by the microlymphocytotoxicity technique10 to verify the absence of HLA antibodies and by standard blood banking techniques to exclude irregular red cell antibodies. Collection and preparation of platelets were as previously described.11 Platelets

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from a donor of the O, blood group were supplied by the American Red Cross (Buffalo, NY) in citrate phosphate dextrose-adenine (CPD-A1) and tested within 24 hours of collection. CPD-anticoagulation and storage at 20 °C is considered optimal for platelet antibody assays when platelets cannot be tested immediately. Group O one-day-old platelets collected in the same anticoagulant gave results not significantly different from those of group O platelets collected in EDTA.

Purification and Radiolabeling of Monoclonal Antibodies

Mouse monoclonal anti-human IgG (Fc) (Bethesda Research Laboratories Inc, Gaithersburg, Md) was purified from ascites fluid and radiolabeled as previously described.11 Mouse monoclonal IgM anti-human type 2H erythrocyte antigen6 was purified from ascites fluid as follows: 2 mL of ascites fluid was precipitated in saturated ammonium sulfate, dissolved in distilled water, and dialyzed at 4 °C for 60 hours in TRIS-HCl (all at pH 8.0). The dialysate was applied to a diethyl aminoethyl (DEAE) cellulose column equilibrated in the same buffer. The protein was then eluted in 0.2 mol/L KCl in 0.01 mol/L TRIS-HCl (pH 8.0) and concentrated before chromatography on Sephadex G-200 with TRIS-NaCl buffer (0.1 mol/L TRIS-HCl, 0.15 mol/L NaCl, and 0.005 mol/L sodium azide). Fractions were pooled and purity was determined by polyacrylamide gel electrophoresis (PAGE). The purified antibody contained minor (less than 5%) contamination with other proteins by sodium dodecyl sulfate (SDS)-PAGE. The antibody was preserved in frozen fractions that were thawed for use and radiolabeled as for the monoclonal anti-human IgG.

Radioimmune Assay

To measure the quantity of type 2H antigen present on platelets, a direct (one-stage) assay system was used to compare the binding of monoclonal anti-type 2H antibody to platelets from different ABO blood group donors. Equal volumes (100 μL) of platelets and 125I-labeled anti-type 2H antibody at a concentration of 500 μg/mL were incubated together for 30 minutes at 22 °C. Anti-type 2H dose-response analysis was performed with doubling dilutions of the labeled anti-type 2H antibody, beginning at a concentration of 2 mg/mL in gelatin-veronal buffer-ethylene diamine tetracetic acid (GVB-EDTA) and incubated at 22 °C for 30 minutes. Incubations were conducted using platelets from group O, A, B, A, B, and O blood type donors.

For the adsorption-elution studies, 200 μL of platelet suspension was incubated for varying time periods in 1 mL of plasma or buffer at room temperature. Uptake of soluble A and B substances was evaluated by mixing group O platelets with group A, and B plasma of known Lewis phenotypes. Loss of A and B antigens from platelets was measured by suspending platelets from group A, and B donors in O plasma, buffer, and autologous plasma, while identical elution studies were done using the group O platelets that had passively acquired A or B antigens by incubation in A, or B plasmas. For these studies, the group O plasma was repeatedly absorbed with A, or B red cells to remove all detectable anti-A or anti-B. Although incubation at 37 °C and 22 °C gave equivalent results, the background levels were higher at 37 °C; therefore, 22 °C was used for all incubations. The platelets were then washed three times in GVB-EDTA and incubated with 200 μL of IgG anti-A or anti-B for 30 minutes at room temperature. After three more washes in GVB-EDTA, 100 μL of platelet suspension was incubated with 100 μL of 125I-labeled monoclonal anti-human IgG for 30 minutes.

After the final incubation in both one- and two-stage assays, platelets were thoroughly resuspended and three 50-μL volumes were pipetted into separate 400-μL microfuge tubes containing a mixture of phthalate esters (1.5 parts n-butyl phthalate [Fisher Scientific Co, Fair Lawn, NJ] and 1.0 part bis [2-ethylhexyl] phthalate [Eastman Kodak Co, Rochester, NY]). After centrifugation in a Beckman (Irvine, Calif) microfuge, a clamp was applied to the tube in the middle of the oil layer, and the tip of the tube was cut off. Each platelet button in the tip, free of unbound 125I-labeled anti-IgG, was counted in a well gamma scintillation counter (Packard Tri-carb, Downers Grove, Ill) for one minute. For each antibody-platelet reaction tested, molecules of IgG bound per cell, r, were calculated using the formula:

\[ r = \frac{6.02 \times 10^{20} \times C_{\text{ion}}/(n)(S_A)}{190} \]

where \( S_A \) is the specific activity in cpm/m mole of the ligand, \( n \) is the number of cells and \( C_{\text{ion}} \) is the cpm of the tips from each sample after correction for the tube background counts. The molar concentration is based on a molecular weight of 1.65 \times 10^5 daltons.13 This assay system is sensitive and specific over a wide range of antigen and antibody concentrations.14

In the figures, "molecules bound per cell" refers to the binding of 125I-labeled anti-type 2H (Figs 1 and 2) and to the binding of 125I-labeled anti-human IgG to cells exposed to IgG anti-A or anti-B (Figs 3 through 5).

RESULTS

In a series of experiments, the reactivity of anti-type 2H antibody with platelets paralleled that predicted for red cells of the same phenotype (Fig 1). Single-factor analysis of variance gave an F value of 190 (P < .0005), while analysis by Student-Newman-Keuls test also demonstrated statistically significant differences in type 2H antigen strength on platelets of different ABO types. The strength of the type 2H antigen on platelets corresponds to the erythrocyte pattern of O > B > A, > A, B > O, phenotypes5-7 and is unaffected by the Lewis and secretor type of the platelet donor. Dose-response curves with anti-type 2H against O, A, and B platelets demonstrate that the type 2H structure is a saturable receptor on platelets (Fig 2).

Group O platelets incubated with A or B plasma acquire A or B antigen reactivity, which increases rapidly during the first hour and then more slowly.

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Fig 1. Binding of 125I-labeled anti-type 2H antibody to platelets from O, B, A, A, B, and O, donors.
Concentration of Antibody (mg/mL)

Fig 2. Dose-response curves for the binding of 125I-labeled anti-type 2H antibody to platelets from O, B, and A1 donors.

from one to 24 hours (Fig 3). Group O platelets incubated in O plasma or buffer show no A or B activity. Group O platelets incubated with A or B plasma for five hours, washed three times, and then incubated in their own original plasma lose 95% of their passively acquired A or B antigen within 18 hours (Fig 3).

Group A1 or B platelets maintained in A or B plasma, respectively, show no change in A or B antigen strength over 24 hours, but A1 or B platelets incubated in buffer or absorbed O plasma demonstrate a decrease in reactivity during the same period. Most of this elution occurs in the first five hours of incubation, and by 24 hours, group A or B platelets still retain significant reactivity as compared with the essentially complete loss of antigens passively acquired on genetic group O platelets (Fig 4). On two occasions we have extended this incubation of group A1 platelets in O plasma to four days with daily changes of plasma. A maximum loss of 45% to 50% of A antigen was reached in approximately two days in group O plasma (Fig 4). In the same experiments, there was no significant change in the level of type 2H antigen present on the

group A1 platelets (dotted line, Fig 4), as measured by a one-stage radioimmunoassay (RIA).

To determine the effect of plasma donor secretor status on the absorption of A and B antigens on O platelets, the platelet suspensions were incubated for 24 hours in A and B plasma from known Lewis-type donors (Fig 5). The difference among the groups is significant when analyzed by Kruskall-Wallis test, although the difference between the Le(a+b-) and Le(a-b+) groups for A absorption is not significant when analyzed by a Wilcoxon two-sample test. The

Fig 3. Adsorption of A antigen onto group O platelets over a 24-hour time period. Group O platelets were incubated in group A1, Le(a-b+) plasma (●), autologous group O plasma (○), or buffer (△). Group O platelets incubated in group A1 plasma for five hours, washed three times in buffer, and then incubated in group O plasma lose most of their acquired antigen (dotted line).

Fig 4. Elution of A antigen from group A1, Le(a-b+) platelets over 96 hours with a change of plasma/buffer every 24 hours. Platelets from a group A, donors (●) were incubated in group O plasma (□), autologous group A1 plasma (○), or buffer (△). The level of type 2H antigen on the same group A platelets is essentially unchanged over the same time period (●).

Fig 5. Molecules of 125I-labeled anti-IgG bound by group O platelets that were exposed for 24 hours to plasma from group A1 (●) and B (○) donors of different Lewis phenotypes and then incubated with anti-A or anti-B.

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coproteins or glycolipids. The terminal immunode-
ant amount of blood group A substance on the platelet is
A or B individuals, most of the A or B antigen on
blood group antigens exist either as type I or type 2 chains. In
fl-D-galactose with an 1-fucose at its two-carbon posi-
tion. The fl-D-galactose is attached to the oligosacchar-
ide backbone by N-acetylglucosamine. This linkage
between $\beta$-D-galactose and N-acetylglucosamine oc-
curs in two forms, a $\beta$(1-3) linkage designated type I
chain and a $\beta$(1-4) linkage termed type 2 chain. Thus
the precursor H antigen and the derived A and B antigens exist
either as type 1 or type 2 chains. In
group A or B individuals, most of the A or B antigen on
ererythrocytes is synthesized by red cell precursors as
type 2 glycolipids, whereas some and perhaps all ABH
antigen passively acquired from the plasma consists of
type 1 glycolipid. Because the H antigen serves as
precursor for A and B antigens, the strength of H
reactivity varies inversely with the amount of A or B
antigen present on the cell, with intrinsic ABH struc-
tures composed of the $\beta$(1-4) type 2 chain.

Our present study demonstrates that approximately
50% to 55% of ABH antigens on platelets occur as type
2H chains, which are presumably intrinsic, as when
found on red cells, and the remainder occur as pas-
sively adsorbed structures, which are presumably type
1H chains of soluble glycolipid. The mouse monoclonal
IgM anti-type 2H antibody has been characterized
previously as specific for the type 2 configuration of
intrinsic red cell H antigen. Direct assays using this
anti-type 2H show dose-response saturation curves
and the predicted variability in strength as an inverse
function of substrate conversion, with H-negative (O) platelets demonstrating essentially no reactivity above
indirect assay background values. If the platelet and
red cell are assumed to be analogous with respect to
derivation of type 2H structures, then our findings
demonstrate that the human platelet possesses intrin-
sic antigens of the ABH system.

Our studies agree with those of previous workers on
the passive uptake of soluble A antigen, which is
probably a type 1 structure. Using the sensitive
RIA, we have extended these observations to include
the adsorption and elution of both A and B antigens on
group O platelets, which is affected quantitatively by
the Lewis and secretor type of the donor plasma.
Furthermore, this rapid passive uptake appears com-
pletely reversible by returning the platelets to their
original O plasma, indicating the gradual loss of the
acquired A substance, presumably owing to elution
from the platelet into the suspending plasma. By
contrast, platelets from genetic A or B donors lost
slightly less than half their reactivity when subjected to
similar incubations, suggesting that a significant por-
tion of their A or B antigens is intrinsic to the cell.

These data suggest that the ABH antigens on
human platelets are a mixture of both intrinsic (type
2H) and extrinsic (type 1H) substances. Variations in
the quantity of both adsorbed and intrinsic antigen
may affect the outcome of ABO incompatible platelet
transfusions, accounting for the irregular initial recov-
eries and distinct biphasic survival curves seen in some
patients. Further work is needed for precise biochem-
ical characterization of platelet ABH antigens and for
assessment of the significance of intrinsic vs adsorbed
antigens in platelet transfusion therapy.

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