Binding of Collagen α1 Chains to Human Platelets

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ABSTRACT We previously reported that purified α1 chains of type I chick skin collagen induce platelet aggregation. We now describe immunological and biochemical evidence that the peptide binds to intact platelets as an early event in the induction of platelet aggregation and the release reaction.

Antibody against α1 (I) was obtained by immunizing rabbits with complete Freund's adjuvant mixed with purified α1. Immunofluorescence studies showed that α1(I)-treated platelets exhibited strong immunofluorescence. The intensity of fluorescence was markedly decreased by the pretreatment of platelets with α1-CB5 and glucosylgalactosylhydroxylysine.

Dose-response curves of platelet aggregation induced by α1 and the binding of α1 by washed intact platelets are correlated. The biochemical studies showed that the binding of the α1 chain to washed intact platelets was platelet concentration and temperature dependent, and that it reached a maximum in 10 min. The process was reversible and specific, with an association constant of 1.7 μM. The inhibitor of α1-induced platelet aggregation, glucosylgalactosylhydroxylysine, inhibited the α1 binding. These results suggest that α1(I) chains bind to specific receptor site(s) on platelet membranes to trigger aggregation and the release reaction.

INTRODUCTION

Platelets play an important role in hemostasis and in pathologic thromboses. After blood vessel injury, platelets adhere to the exposed subendothelial connective tissue, collagen in particular (1–5), and aggregate, releasing several biologically active substances. Collagen and other agents, such as ADP, thrombin, epinephrine, and antigen-antibody complexes, have been shown to induce platelets to aggregate and undergo the "physiologic" release reaction in preparations of platelet-rich plasma (6–13).

It has been suggested that these aggregating agents may initiate these phenomena by acting on the platelet surface. Thus, it has been shown that ADP binds to isolated platelet membranes (14), and that thrombin binds to the membrane of intact platelets (15, 16). The mechanisms whereby collagen may mediate platelet aggregation and the structural features responsible for the interaction have not been completely clarified. Previously, we reported that the purified, denatured α1(I) chain of chick skin collagen is capable of causing platelet aggregation and the release reaction, and that the carbohydrate moiety located in one of the CNBr peptides, α1(I)-CB5, is involved in the interaction (17–19). Inhibition of adenylate cyclase of platelet membranes by collagen and the α1 chain (19) suggested the possibility that these substances act directly on the platelet suface. In the present paper, we report evidence to indicate that α1 chains bind specifically and reversibly to the intact platelet surface, and that the sugar moiety, glucosylgalactosylhydroxylysine (Glc-Gal-Lys[OH]),[1] is involved in the interaction. The close correlation between the degree of binding and aggregation supports the hypothesis that the binding of α1 to membrane receptor(s) may be an initial event which culminates in platelet aggregation.

METHODS

Preparation of washed human platelets. Blood was collected from normal volunteers after they had fasted overnight and placed in polypropylene tubes containing 0.1 vol of 3.8% sodium citrate. Platelet-rich plasma was prepared by centrifuging the citrated blood at room temperature for 15 min at 226 g (18). The contaminating erythrocytes were removed by centrifugation at 1,500 g for 1 min. The erythrocyte-free platelet-rich plasma was then spun at 2,000 g for 15 min in the presence of 0.001 M EDTA. The platelets

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[1] *Abbreviations used in this paper*: Glc-Gal-Lys(OH), glucosylgalactosylhydroxylysine; PBS, 0.02 M phosphate/0.15 M, NaCl, pH 7.4; Tris-EDTA, 0.01 M Tris/0.001 M EDTA per 0.15 M NaCl, pH 7.5.

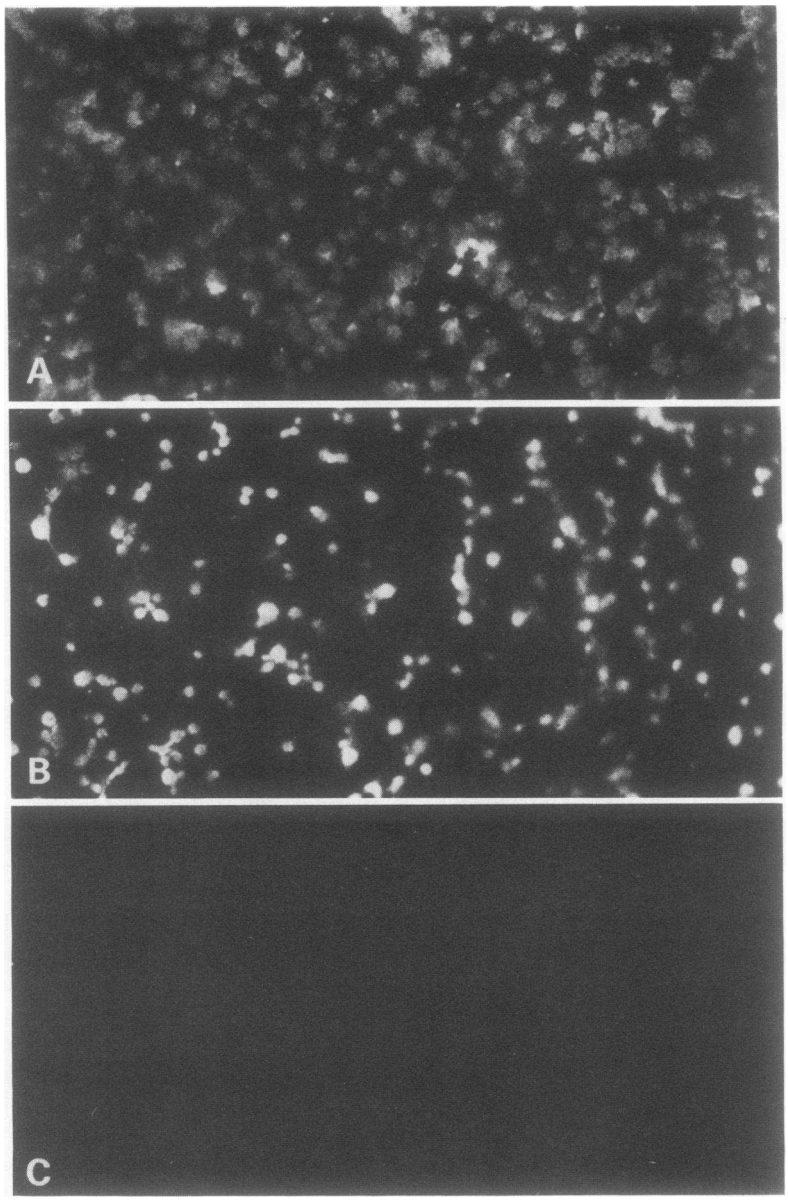

FIGURE 1 Human platelets treated with α1(I) chain (A) or soluble tropocollagen (B) prepared from chick skin followed by rabbit anti-α1 or antitropocollagen, respectively, and then by fluorescein-conjugated goat antirabbit IgG. Preincubation of platelets with Glc-Gal-Lys(OH) before incubation with α1 chain markedly reduced immunofluorescence (C) which was comparable to control platelets incubated with PBS rather than α1 or tropocollagen.

were washed twice with 0.01 M Tris/0.001 M EDTA per 0.15 M NaCl, pH 7.5 (Tris-EDTA), and then resuspended in the same buffer at the concentration of 300,000–400,000/mm³. The washed platelets were kept in an ice bath until used. The experiments were performed within 3 h after collection.

Preparation of chick skin collagen, α1 chains, and CNBr peptides. Neutral salt-soluble collagen was extracted from the skin of 3-wk-old, White Leghorn chicks which had been rendered lathyritic by administration of β-aminopropionitrile (Aldrich Chemical Co. Inc., Milwaukee, Wis.) for 2 wk. Extracted collagen was purified by repeated differential precipitation with NaCl as previously described (20). The α1 chain was prepared from purified lathyritic collagen by chromatography on carboxymethylcellulose (20). For preparation of radiolabeled α1, the animals had been injected intraperitoneally with 100 μCi of [^{14}C]glycine (sp act, 49.73 μCi/mmol, New England Nuclear, Boston, Mass.) in 1 ml of sterile 0.9% NaCl once a day for 3 days before they were killed.

The CNBr peptides of the α1 chain were prepared by a combination of ion-exchange and molecular-sieve chroma-

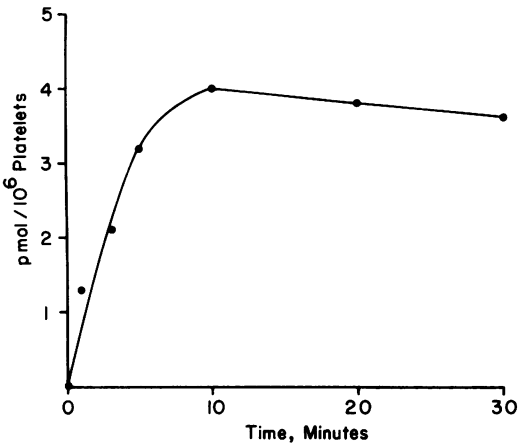

FIGURE 2 Time-dependent binding of ^{14}C-labeled chick skin α1 by platelets. 25 μg of ^{14}C-labeled α1 was incubated with the washed platelets (1×10^6) at 37°C. At the time indicated, platelet-bound ^{14}C-labeled α1 was isolated by Millipore (Millipore Corp.) filtration. Each point is the mean of duplicates. All values were corrected for nonspecific binding.

tography of CNBr digests of the α1 chain on various resins. The details of these procedures as well as the criteria of their purity have been described in detail elsewhere (21).

Preparation of antiserum against α1 chain and collagen. Antisera were raised in rabbits immunized with 1 mg of collagen or α1 chain emulsified in 1.0 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). 4 wk later, the animals were boosted with 1 mg of alum-precipitated (22) collagen or α1 chain injected intraperitoneally at weekly intervals for 3 wk and again on wk 10, followed by an intravenous injection of the same dose on wk 11. The rabbits were bled 7 days after the last injection. Sera were stored at 4°C. Antibody titers were assayed by passive hemagglutination of tanned human group O erythrocytes coated with collagen or α1 chain (23). The antibodies were partially purified by quarternary-aminoethyl-Sephadex (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) column chromatography as previously described (24).

Immunofluorescence studies. Smears of washed platelets were prepared on glass microscope slides. The dried smears were washed with 0.02 M phosphate/0.15 M NaCl, pH 7.4 (PBS), and fixed with 1% paraformaldehyde for 1 min. The washed, fixed smears were treated with 1-mg/ml solutions of collagen or α1 chain in PBS for 20 min at room temperature, washed with PBS twice, incubated with antiserum either to collagen or α1 chain for 20 min at room temperature, washed twice again with PBS, and finally treated with a 0.1 dilution of fluorescein-conjugated, goat anti-rabbit IgG for 20 min. At the end of incubation, the smears were washed with PBS and mounted with glass coverslips using one drop of Gelvatol (Monsanto Co., St. Louis, Mo.). The smears were examined with a fluorescence microscope (Carl Zeiss, N. Y.).

Binding experiments. Assays for the binding of the ^{14}C-labeled α1 chain to washed platelets were performed in Tris-EDTA at various temperatures. The assay mixtures consisted of 25 μg of [^{14}C]glycine-labeled α1 chain in 0.1 ml of Tris-EDTA and various amounts of washed platelets for various periods of time as indicated in the text. In some experiments, various other proteins or peptides were added to the mixtures as specified. After specified intervals, incubations were terminated by passing the mixtures through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) under reduced pressure. The platelets were washed with 10 ml of 0.05 M Tris, pH 7.5, containing 0.5% bovine serum albumin, and counted in a Nuclear-Chicago scintillation counter (Nuclear-Chicago, Des Plaines, Ill.) using Aquasol (New England Nuclear) as solvent.

Identification of platelet-bound radioactivity. The bound ^{14}C-labeled α1 chain was dissociated by soaking the filter in 20 ml of 0.05 M Tris, pH 7.5, containing 1.25 mg/ml of unlabeled α1 at 37°C for 1 h with constant shaking. Approximately 80% of the total radioactivity was recovered in the extract.

A portion of the lyophilized extract was dissolved in 2 ml

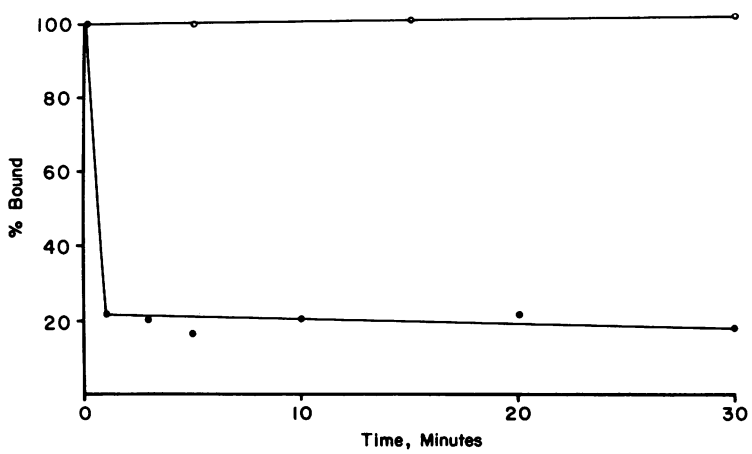

FIGURE 3 Dissociation of platelet-bound 25 μg of ^{14}C-labeled α1 was preincubated with the washed platelets (6×10^6) in 100 μl of Tris-EDTA buffer for 30 min at 37°C. At 0 time, 1.25 mg of unlabeled α1 dissolved in 0.2 ml of the same buffer was added and incubated for the additional indicated times (●). To the controls was added 0.2 ml of the buffer only (○). Each point represents separate duplicate incubations.

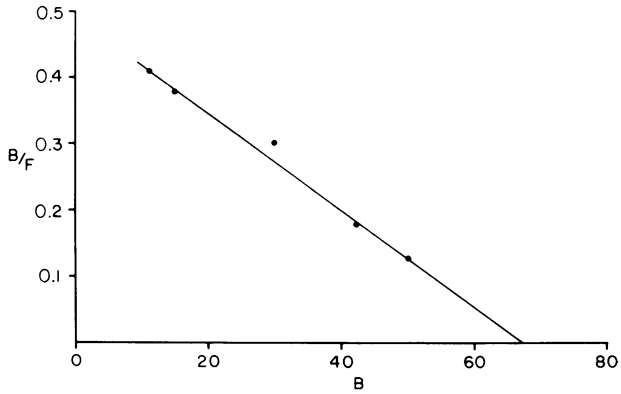

FIGURE 4 The ratio, bound/free ^{14}C-labeled α1, is plotted as a function of bound α1 according to the method of Scatchard (26). B, amount of α1 bound (pmol/7 × 10^7 platelets) to platelets; F, amount of free α1 in micromoles. The intercept with the abscissa, 67 pmol/7 × 10^7 platelets, corresponds to an approximation of the binding capacity of platelets at saturation. From the Avogadro number (6.02 × 10^{23}), the number of binding sites per platelet was calculated to be approximately 600,000.

of 0.01 M Tris/1 M $CaCl_2$, pH 7.4, applied to a 2 × 110-cm column of agarose A 1.5 m (Bio-Rad Laboratories, Richmond, Calif.), and eluted with the same buffer. 3-ml fractions were collected. The remaining extract was dissolved in 5 ml of 0.05 M sodium acetate, pH 4.8, together with 10 mg of unlabeled α1 to serve as carrier. The mixture was applied to a 2 × 10-cm column of carboxymethylcellulose which had been equilibrated with the same buffer at 43°C. The column was eluted with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 800 ml and 13-ml fractions were collected. The optical density (230 nm) of each of the effluent fractions was determined using a Zeiss spectrophotometer and the radioactivity was assayed in the scintillation counter.

Determination of protein concentration. The concentrations of collagen-derived peptides were determined by hydroxyproline analyses after hydrolysis as described previously (25). The platelet counts were performed by phase-contrast microscopy.

RESULTS

Immunofluorescence studies. Evidence that α1 chain of collagen binds to the intact human platelet was obtained by immunofluorescence studies. The α1 chain-treated platelets fluoresced intensely and uniformly (Fig. 1A) whereas tropocollagen-treated platelets fluoresced unevenly (Fig. 1B) as compared to the absence of fluorescence in control platelets treated with PBS. The intensity of immunofluorescence produced by α1 and collagen was markedly reduced by preincubation of platelets with α1-CB5 or Glc-Gal-Lys(OH) (Fig. 1C), but not with other CNBr peptides, including CB4, CB7, and CB8, suggesting that Glc-Gal-Lys(OH) is a determinant in the binding of α1 by platelets.

Binding of α1 chain to washed human platelets. The binding of α1 chain to platelets is time (Fig. 2) and platelet concentration dependent (data not shown). The binding of α1 chain to platelets is also temperature dependent; the total amount bound to platelets at 37°C is three to four times higher than that at room temperature and 0°C (data not shown). In addition, the rate of binding at 37°C exceeded that at room temperature and 0°C. In subsequent studies therefore, the binding experiments were performed at 37°C. The amount bound at various temperatures was corrected for the background using the same amount of ^{14}C-labeled α1 chain at parallel points in the absence of platelets.

The binding of the α1 chain to platelets was shown to be a reversible process (Fig. 3). The bound α1 chain was rapidly displaced from platelets by adding a 50-fold excess of unlabeled α1 chain. The undisplaceable radioactivity was considered to represent nonspecific binding. In subsequent experiments, therefore, duplicate incubations were performed with and without the addition of 50-fold excess of unlabeled α1 chain. Each value of binding was corrected by subtracting the value obtained in a duplicate incubation mixture containing excess unlabeled α1.

Determination of association constant. To determine the average number of α1-chain receptor sites per platelet as well as their affinity, the amount of α1

TABLE I
*Effects of α1 CNBr Peptides, Glu-Gal-Lys(OH), and Hydroxylysine on ^{14}C-Labeled α1 Binding by Platelets**

Test substances	Control
	%
Control	100
α1, 125 μg	43
α2, 125 μg	40
β12, 125 μg	61
CB4, 125 μg	106
CB5, 125 μg	65
CB5, 250 μg	41
CB7, 125 μg	97
CB8, 125 μg	84
BSA‡, 125 μg	110
Glc-Gal-Lys(OH), 125 μg	79
Glc-Gal-Lys(OH), 250 μg	52
Hydroxylysine, 125 μg	115
Hydroxylysine, 250 μg	104

* Washed platelets (6 × 10^6) were incubated with the CNBr peptides or Glc-Gal-Lys(OH), or hydroxylysine at 37°C for 20 min. After the addition of 25 μg of ^{14}C-labeled α1, the mixtures were incubated at 37°C for an additional 20-min period. The bound α1 was isolated by Millipore filtration. Values are expressed as the percent of control experiments.
‡ BSA, bovine serum albumin.

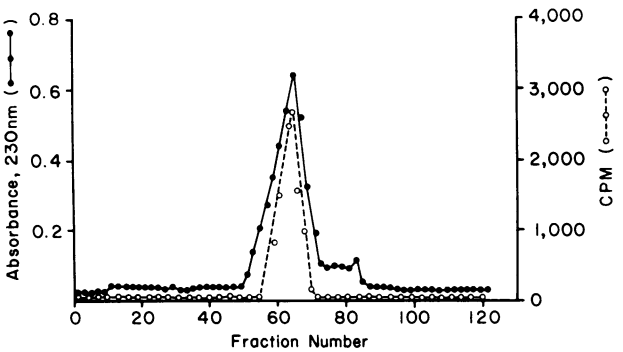

FIGURE 5 Agarose A 1.5 m column chromatography of α1 chains displaced from the washed platelets which had been incubated with ^{14}C-labeled α1. The radioactivity was displaced with a 50-fold excess of unlabeled α1. The dissociation material was lyophilized and redissolved in 0.01 M Tris-1 M $CaCl_2$, pH 7.4, applied to a 2 × 110-cm column, and eluted with the same buffer. Aliquots of the column effluent fractions were analyzed for radioactivity (○) and optical density at 230 nm (●).

bound to platelets was determined as a function of free α1 added to the system. The resulting data were analyzed by a Scatchard plot (26) (Fig. 4). The number of molecules bound to platelets at saturation was calculated from the intercept with the abscissa. From a number of experiments using different platelet sources, it was determined that there was a single population of approximately 600,000 binding sites per platelet with an association constant of 1.7 μM.

Inhibition studies. In an attempt to identify the interaction site(s) between α1 chain and platelets, Glc-Gal-Lys(OH) and α1-CB5 were tested for their ability to inhibit binding of α1 to intact platelets. The results summarized in Table I demonstrate that these two substances inhibit binding of the α1 chain to platelets. In contrast, the other collagen peptides, CB4, CB7, CB8, as well as hydroxylysine and bovine serum albumin had no effect on the binding of α1.

Identification of the displaced α1 chain. To determine whether α1 was altered or whether it remained unaltered after it became bound to the intact platelet, ^{14}C-α1 chains were first reacted with platelets, and then displaced with a 50-fold excess of unlabeled α1. The dissociated material was mixed with unlabeled, carrier α1 and chromatographed on a column of agarose A 1.5 m. Only one radioactive peak which coeluted with the unlabeled α1 chain was observed (Fig. 5). Recovery of the radioactivity from the chromatogram was 90%. No evidence of heterogeneity in radioactive material was observed. Similar results were obtained by carboxymethylcellulose chromatography of a separate aliquot (figure not shown). These results indicate that the α1 chain remained intact during the binding and displacement reactions.

Relationship of the α1 binding with platelet aggregation. The various concentrations of α1 chain used to induce platelet aggregation (17–19) together with the amounts of α1 bound to washed intact platelets at each concentration of α1 are depicted in Fig. 6. The dose-response curve of α1 chain-induced platelet aggregation demonstrates a direct relationship with α1 chain binding to platelets. A relatively small amount of binding is associated with the functional

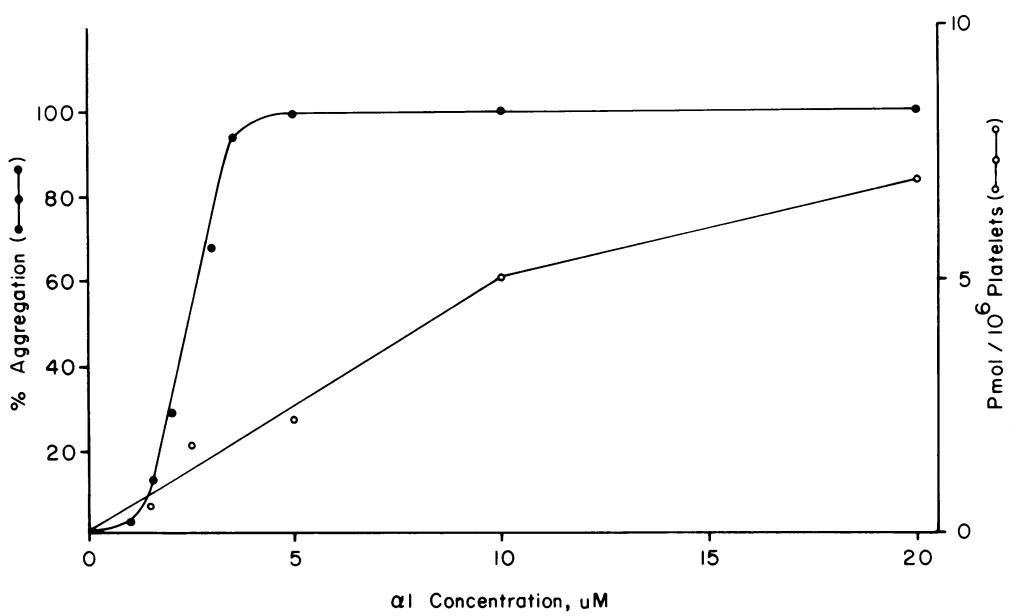

FIGURE 6 Comparison of platelet aggregation induced by α1 (●) and the binding of ^{14}C-labeled α1 by washed platelets (○).

response, aggregation. A similar relationship has been observed between hormone binding such as insulin and the biological response of the target cells (27).

DISCUSSION

The immunofluorescence studies reported here indicate that collagen and the α1(I) chains of chick skin bind to the surface of platelets, and that the binding is inhibited by Glc-Gal-Lys(OH), a known inhibitor of α1 chain-induced platelet aggregation (17, 18). These observations were confirmed by biochemical studies which indicate that binding of α1(I) to intact platelets is reversible and specific as evidenced by the fact that ^{14}C-labeled α1 chain can be displaced from intact washed platelets by unlabeled α1. Scatchard plot analyses suggest that platelets possess a single population of binding site(s) with an association constant of 1.7 μM for the interaction with α1, and that at saturation an average of 600,000 molecules of α1 are bound per platelet. The observed association constant is consistent with the amount of α1 necessary to induce platelet aggregation and the release reaction (19). However, it is still possible that there may have been an additional population(s) present in platelets as has been shown in thrombin-binding experiments (15). Because of the limitations in obtaining α1 chain of sufficiently high specific activity by in vivo labeling, it was impossible to examine binding at much lower concentrations of α1 than used in the present study.

Inhibition studies using various collagen-derived peptides indicate that Glc-Gal-Lys(OH) and the glycopeptide, α1-CB5, interfere with the α1 binding. In contrast, free hydroxylysine, and other CNBr peptides not containing the hydroxylysineglycoside, have no effect. These results are consistent with previously reported findings that Glc-Gal-Lys(OH) inhibits α1-induced platelet aggregation (17, 19) but not aggregation induced by other agents such as ADP (unpublished observations).

Platelet aggregation occurred after binding of relatively small quantities of α1 to platelets. This is consistent with the relationship reported previously for insulin binding and the biological response of fat cells (27). These observations strongly support the idea that the collagen α1 chain induces platelet aggregation by acting on the platelet membrane. It should be noted, however, that these studies have been carried out using radiolabeled chick skin α1 chains for cogent technical reasons, and that the reactivity of human platelet membrane to collagens of other sources may differ (17, 18). Further studies to identify the receptor sites on platelet membranes may provide useful information both in regard to platelet variations and in regard to collagen abnormalities in various disease states manifested by abnormal collagen-platelet interactions.

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