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A COMPARATIVE STUDY OF THE EFFECT OF MODIFICATION OF THE SURFACE OF HUMAN PLATELETS ON THE RECEPTORS FOR AGGREGATED IMMUNOGLOBULINS AND FOR RISTOCETIN-VON WILLEBRAND FACTOR

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Summary

The receptors for aggregated immunoglobulin G (IgG) (an Fc receptor) and for ristocetin-von Willebrand factor on human platelets were studied by means of various modifications of the platelet surface. The expression of these receptors was measured by the agglutination of platelets to ristocetin in the presence of von Willebrand factor, which is part of the factor VIII complex, and by the binding of aggregated IgG coupled to ^{3}H-labelled diazobenzene. Treatment of platelets with chymotrypsin, trypsin, papain and pronase which removed protein and glycoprotein from the platelet under conditions where the release reaction was inhibited caused loss of the expression of the receptor for ristocetin-von Willebrand factor and an enhancement of that for aggregated IgG. Induction of membrane changes with ADP and of the release reaction with the ionophore A23187 abolished agglutination to ristocetin-von Willebrand factor but did not alter the receptor for aggregated IgG. Possible contributions of unspecific membrane changes, produced by protease treatment of platelets, to the modification of receptor expression were eliminated by the use of formaldehyde-treated platelets. Trypsin, papain and pronase destroyed the ability of these platelets to agglutinate to ristocetin-von Willebrand factor but produced no change in the binding of aggregated IgG. Therefore, the receptor for ristocetin-von Willebrand factor is truly sensitive to proteolysis while the Fc receptor is not, but is partially masked by protease-sensitive material.

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Abbreviations: IgG, immunoglobulin G; ^{3}H-labelled DB.BDB-IgG, IgG aggregated by coupling with bis-diazobenzidine and labelled with [G-^{3}H]diazobenzene.

Introduction

Antigen-antibody complexes and aggregated immunoglobulins of the class G (IgG) can react with washed human platelets, inducing release of intracellular granule contents and cell aggregation [1—3]. The interaction of aggregated IgG with platelets [4,5] like that with other blood cells [6—9], appears to occur via an Fc receptor. The nature of this receptor on platelets is not known, but there is some evidence that in murine lymphocytes it is a glycoprotein [10]. Studies of the binding of IgG aggregates to platelets, using as a model IgG aggregated by coupling with bis-diazobenzidine (BDB-IgG) and labelled with [^{3}H]diazobenzene (^{3}H-labelled DB.BDB-IgG) [11] showed that binding of 40—70 BDB-IgG aggregates per platelet was sufficient to induce the release reaction. Thus binding of BDB-IgG, rather than the cell response, appeared to be a convenient and sensitive means of investigating the nature and location of the platelet Fc receptor.

The nature of surface receptors of platelets has been the subject of considerable recent attention and glycoproteins have been strongly implicated [12—25]. In particular, glycoproteins appear to be important in the response of platelets to von Willebrand factor, which is part of the Factor VIII complex [26], in the presence of the antibiotic ristocetin [12,13]. This response appears to parallel the ability of the platelet to adhere to blood vessel walls in the initiation of haemostasis [14,15]. Platelets from patients with Bernard Soulier syndrome show defective adhesion and do not agglutinate to ristocetin. In addition their membrane glycoprotein content and exposure are abnormal [12,13]. Normal platelets treated with chymotrypsin or with an uncharacterised protease from *Nocardia lurida* also have a decreased glycoprotein content and show a reduced response to ristocetin [12].

It was of interest to observe that platelets fixed with formaldehyde, although unable to undergo the release reaction in response to aggregation-inducing agents [27], still agglutinate with ristocetin-von Willebrand factor [27,28] and also bind aggregated IgG [11]. We therefore initiated studies to examine whether the receptors for aggregated IgG and those for ristocetin-von Willebrand factor were in any way related, comparing their expression on platelets which had been modified by proteolytic enzymes.

Studies of surface receptors of platelets are, however, complicated by the fact that proteases other than chymotrypsin and that from *Nocardia lurida* may, in addition to removing surface proteins and glycoproteins [12,16,17, 29—31] cause the release reaction [32] which is accompanied by marked membrane alterations as indicated by increased exposure of lectin-binding sites [33]. In addition, recent findings have shown that treatments of platelets much milder than proteolysis alter the expression of the receptor for ristocetin-von Willebrand factor. ADP and serotonin in concentrations insufficient to induce release inhibit ristocetin-induced agglutination [34,35]. Formaldehyde-fixed platelets, however, responded normally to ristocetin-von Willebrand factor in the presence of ADP and serotonin. It was suggested that the transformation by low concentrations of ADP or serotonin of a normal discoid platelet to a sphere with many pseudopodia (the "shape change") alters the platelet membrane so that the receptor for ristocetin-von Willebrand factor is no longer

available. Thus the failure of chymotrypsin-treated platelets to respond to ristocetin could have been due to similar secondary membrane changes following proteolysis rather than to actual destruction of the receptor.

We have therefore attempted not only to minimise such secondary membrane changes by the use of release inhibitors, but to ascertain to what extent they may cause any observed alteration in the expression of receptor activity. We have compared receptor expression on platelets treated with proteases, ADP or the ionophore A23187, which induces release by directly altering membrane permeability to Ca^{2+} [36—39] and have also examined the effects of proteolysis on formaldehyde-fixed platelets.

Materials and Methods

Human blood platelets. These were isolated within 20 h after collection from citrated blood collected for the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross in Berne [40]. The buffy coats were syphoned into a buffered glucose solution; the resulting platelet-rich plasma contained about 20 mM glucose, 12 mM phosphate buffer, pH 6.8, and $3 \cdot 10^9$—$4 \cdot 10^9$ platelets per ml. Platelets were washed by repeated centrifugation (9 min, 2200 $\times g$, room temperature) and resuspension. The washing solution consisted of 0.14 M NaCl, 5.5 mM glucose, 5 mM EDTA, 10 mM Tris · HCl, pH 7.4. The platelets were finally suspended in this buffer and their concentration determined by a nephelometric method.

Labelled compounds. These were obtained from the Radiochemical Centre, Amersham (U.K.) if not stated otherwise. [^{14}C]Serotonin (CFA. 170) was dissolved in 70% ethanol to 8 μCi/ml and 0.15 mM. [^{3}H]Adenine (TRK. 23) was used as an aqueous solution of 0.5 mCi/ml and 0.5 mM. (Hydroxy[^{14}C]-methyl) inulin was dissolved in 0.15 M NaCl to a concentration of 1 μCi/ml and 0.1 μM. [G-^{3}H]Aniline · HCl (NET. 052) was obtained from New England Nuclear Chemicals GmbH, Dreieichenhain (Germany) as a solution in ethanol with 10 mCi/ml and 0.1 M.

Proteases. Trypsin, Type I from bovine pancreas and twice recrystallised papain were obtained from Sigma, St. Louis, Mo. (U.S.A.). Pronase was obtained from Calbiochem. Los Angeles, Calif. (U.S.A.) and chymotrypsin from Worthington Biochemical Corp., Freehold, N.J. (U.S.A.). With the exception of papain, these enzymes were dissolved immediately before use in 0.14 M NaCl, 5 mM glucose, 5 mM EDTA, 10 mM Tris · HCl, pH 7.4. Papain was dissolved in 0.15 M Tris, pH 7.6, containing 2 mM cysteine. Their activity was measured by the proteolysis of 3% (w/v) casein. After incubation for 30 min at 37° C, 1.5 vol. of cold 10% trichloroacetic acid were added and after 30 min at 0° C, the precipitated protein was removed by centrifugation and the acid-soluble peptides in the supernatants after neutralisation were measured by their absorbance at 280 nm, using undigested casein as standard.

Other substances. Ristocetin, Lot 3003-109-30, was from Abbott Laboratories, North Chicago (U.S.A.). It was stored at —20° C as a stock solution in 0.15 M NaCl containing 200 mg/ml. The stock solution was diluted with 0.15 M NaCl before use. Phenylmethylsulphonylfluoride was obtained from Sigma. The stock solution was 3.5 mg/ml in methanol. It was diluted 5-fold with

buffer before addition to platelet samples (final concentration of methanol, 10% (v/v)). Prostaglandin E_1 was the gift of Dr. J. Pike, Upjohn Co., Kalamazoo, Mich. (U.S.A.). 3.5 mg were dissolved in 0.1 ml ethanol and 0.9 ml 0.2% (w/v) Na_2CO_3 was added. The ionophore A23187 was the gift of Dr. R.L. Hamill, Eli Lilly, Indianapolis, Ind. (U.S.A.). It was dissolved in ethanol and added with a microsyringe to platelet samples (final concentration of ethanol: 0.4% (v/v)). Scintillator was a mixture of two parts of xylene and one part of Triton X-100 containing 3 g/l Permablend III (Packard). Sodium iodoacetate and ADP were obtained from Sigma. All other chemicals were of reagent grade.

Preparation of labelled aggregated IgG. IgG was purified as previously described [41] and aggregated by coupling with *bis*-diazobenzidine [3]. The lyophilised product (BDB-IgG) was suspended at a concentration of 20 mg/ml in 0.15 M NaCl, 0.01 M Tris · HCl, pH 8.0. Insoluble material was sedimented at 3000 × *g* for 15 min and the supernatant fractionated on Biogel A-5 m at 2°C. The buffer used for equilibration of the column and elution was 0.15 M NaCl, 0.01 M Tris · HCl, pH 8.0. The material eluting in the void volume was concentrated by pressure dialysis and stored at −70°C. The labelling procedure was essentially that used in the initial coupling reaction. To 10 ml solution of BDB-IgG (5 mg/ml) at 0°C was added 1 ml saturated $Na_2B_2O_7$ and a mixture containing 40 µl [G-^{3}H]aniline · HCl (400 µCi), 50 µl 0.25 M KCl and 10 µl 0.5 M $NaNO_2$ (which produced [G-^{3}H]diazobenzene). After 30 min, the solution was loaded on a Sephadex G-25 column and the protein eluting in the void volume was extensively dialysed against 0.15 M NaCl, 0.01 M Tris · HCl, pH 8.0 at 2°C. Insoluble material was removed by centrifugation at 38 000 × *g* for 30 min at 2°C and the supernatant stored at −70°C. Such preparations of IgG aggregated by coupling with *bis*-diazobenzidine and labelled with [G-^{3}H]diazobenzene contained approx. 0.3 diazobenzene molecule per IgG and 10—30 IgG molecules per aggregate [11] and are termed ^{3}H-labelled DB.BDB-IgG. Before use this preparation was diluted to 0.32 mg/ml with a solution of 1 mg/ml bovine serum albumin in 0.15 M NaCl.

Treatment of platelets with formaldehyde. Platelet-rich plasma was incubated at 37°C for 30 min, mixed with an equal volume of 2% formaldehyde solution (Merck, Darmstadt, G.F.R.) in buffer containing 0.14 M NaCl, 5 mM EDTA, 5.5 mM glucose and 0.01 M Tris · HCl, pH 7.4 and stored at 2°C for 18 h as described by Kirby and Mills [27]. After centrifugation the platelets were washed three times in the above buffer from which the glucose had been omitted and stored at 2°C in the presence of 0.05% (w/v) NaN_3.

Treatment of platelets with proteases. Platelets were washed until they no longer agglutinated in the absence of added von Willebrand factor (6—7 times) as described above and resuspended to a concentration of $3 \cdot 10^9$ per ml. To 20 ml of this suspension were added either 2 ml 10 mM prostaglandin E_1 and 2 ml 10 mM acetylsalicylic acid to inhibit the release reaction or 4 ml 0.15 M NaCl. After 30 min incubation at room temperature 3-ml portions were incubated with 0.3 ml enzyme solution or buffer at 37°C for 10 min. The final enzyme concentrations were: chymotrypsin, 200 µg/ml; trypsin, 50 µg/ml; pronase, 50 µg/ml; papain, 25 µg/ml. The activation of papain was ensured by including cysteine to a final concentration of 0.2 mM. To the papain-containing mixture was then added 3.3 ml 0.02 M sodium iodoacetate, 0.15 M Tris · HCl,

pH 7.6; to all other mixtures were added 3.3 ml 4 mM phenylmethylsulphonylfluoride to stop enzyme action [42]. After a further 10 min at 37°C, the platelets were centrifuged, washed twice in washing buffer and resuspended to the desired concentration.

Measurement of the release reaction. Platelets were labelled by preincubating the platelet suspension or platelet-rich plasma, both containing $3 \cdot 10^9$ platelets per ml, with [^{14}C]serotonin (0.3 μM) and [^{3}H]adenine (0.25 μM) for 1 h at 18°C as described [43]. After the appropriate incubation in the test system being examined, the platelets were centrifuged in a microcentrifuge (20 s, 11 000 × *g*). An aliquot of the supernatant (0.1 or 0.2 ml) was diluted to 1.1 ml with 0.5% (w/v) Triton X-100, mixed with scintillator and counted in a dual channel liquid scintillation spectrometer (Packard). The percent release of [^{14}C]serotonin was expressed as described [43]. Cytoplasmic leakage, as measured by the appearance of labelled adenine metabolites in the supernatant [44] was expressed in the same way.

Measurement of the binding of ^{3}H-labelled DB.BDB-IgG. For washed or formaldehyde-treated platelets 0.2 ml of a suspension which had been adjusted to $2.5 \cdot 10^9$ platelets per ml were added to a 1 ml polypropylene centrifuge tube which contained 0.4 ml of a medium containing 10 mM EDTA, pH 7.6, 30 mM KCl, 20 mM NaCl, 2 μM imipramine, 6 mg/ml bovine serum albumin and 90 mM Tris · HCl (pH 7.6), 0.05 ml (hydroxy[^{14}C]methyl) inulin (1 μCi/ml), 0.1 ml 1 mg/ml bovine serum albumin in 0.15 M NaCl. For binding to platelets in plasma, 0.5 ml platelet-rich plasma (adjusted to $1 \cdot 10^9$ platelets per ml with platelet-free plasma) was incubated with 0.1 ml 8 μM imipramine in 0.15 M Tris · HCl, pH 7.6, 0.1 ml 0.1 M EDTA, pH 7.6, 0.05 ml (hydroxy-[^{14}C]methyl) inulin (1 μCi/ml). These mixtures all contained $5 \cdot 10^8$ platelets and had a final pH of 7.3—7.4. They were incubated at 37°C for 2 min prior to the addition of 0.05 ml ^{3}H-labelled DB.BDB-IgG (0.32 mg/ml). The suspension was mixed rapidly with a glass rod and incubated for the required time, when 0.15 ml of a mixture of dinonyl phthalate/dibutyl phthalate (1 : 2.56, v/v) [36] was layered over the suspension, the tube stoppered and rapidly inverted, resulting in movement of the organic phase to the bottom, and centrifuged for 20 s at 11 000 × *g*. The platelets formed a sediment separated from the supernatant by the organic phase. 0.2 ml of the supernatant were diluted in Triton X-100 and counted as described above. The remaining liquid in each tube was decanted and the tube inverted and drained. Traces of liquid were removed with tissue paper and the sediment solubilized in 0.05 ml xylene and 0.1 ml 0.5% (w/v) Triton X-100 as described [37]. This solution was poured into a counting vial and the tube rinsed with three times 0.33 ml 0.5% (w/v) Triton X-100. 10 ml scintillator were added and the sample counted as described above. The amount of bound ^{3}H-labelled DB.BDB-IgG, corrected for the adhering and enclosed medium was calculated as follows:

$$\text{cpm } ^3\text{H bound} = \text{cpm } ^3\text{H in sediment} - \frac{\text{cpm } ^{14}\text{C in sediment} \cdot \text{cpm } ^3\text{H per } \mu\text{l of supernatant}}{\text{cpm } ^{14}\text{C per } \mu\text{l of supernatant}}$$

$$\text{and } \mu\text{g } ^3\text{H-labelled DB.BDB-IgG bound} = \frac{\text{cpm } ^3\text{H bound}}{\text{cpm } ^3\text{H per } \mu\text{g } ^3\text{H-labelled DB.BDB-IgG}}$$

Controls with 1 mg/ml bovine serum albumin in 0.15 M NaCl instead of the platelet suspension were used to correct for labelled protein unspecifically absorbed to the plastic tubes. This did not exceed 5% of that bound to $5 \cdot 10^8$ platelets in 5 min.

Ristocetin-induced platelet agglutination. This was measured at 37°C by a nephelometric method using a Labintec aggregometer (Montpellier, France) as described [12]. To 0.2 ml of platelet suspension ($1.5 \cdot 10^8$ platelets per ml) was added 0.04 ml plasma (from normal donors and stored at −20°C) or 0.15 M NaCl. This suspension was equilibrated for 1 min in the aggregometer and 0.01 ml ristocetin solution added. The velocity of agglutination was measured as described [45]. In order to compare experiments on different days, the velocity of ristocetin-induced agglutination in the control sample was given an arbitrary value of 100 and the velocities of the other samples expressed as a percentage. Values are the means of duplicate experiments.

Polyacrylamide gel electrophoresis. Portions of the platelet suspensions were centrifuged (3000 × *g*, 10 min) and the pellets resuspended in 0.2 ml water and solubilised by the addition of 0.1 ml 10% (w/v) sodium dodecyl sulphate and incubation at 100°C for 10 min. Samples of these solubilised platelets (80—100 μl contained 0.6—0.8 mg protein) were analysed in the reduced state by treatment with 50 μl of a mixture containing 5% (w/v) dithiothreitol, 8 M urea, 2% (w/v) sodium dodecyl sulphate in 0.01 M sodium phosphate, pH 7.35. The sample was then heated at 100°C for 3 min and electrophoresed on 5% (w/v) acrylamide gels as previously described [16]. The gels were 13 cm long and contained 0.1% sodium dodecyl sulphate. After electrophoresis the gels were stained either for protein with Coomassie Brilliant Blue or for carbohydrate with periodic acid-Schiff's reagent. To detect the glycoprotein it was necessary to use samples containing 800 μg protein. The gels were scanned in a densitometer (Kipp and Zonen, Delft, Holland) at 550 nm.

Results

Treatment of platelets with proteases

Conditions were first sought under which surface components could be modified by proteolysis with minimal associated release or platelet lysis. 1 mM prostaglandin E_1 and 1 mM acetylsalicylic acid, known to inhibit release by other platelet aggregating agents [46], inhibited both release and lysis caused by treatment of washed platelets with trypsin, pronase and papain (Table I).

Examination of protease-treated platelets by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate showed that considerable amounts of material staining for carbohydrate had nevertheless been removed both in the presence and in the absence of release inhibitors (Fig. 1). Since the larger part of platelet material staining with periodic acid-Schiff's reagent is glycoprotein which is exposed on the surface [16], it was concluded that surface glycoproteins were being modified during treatment with all the proteases examined. In the control samples glycoproteins I, II and III are clearly seen (the nomenclature is that used in previous studies [17,18]). Other glycoproteins that have been described [22] are not visible since only fully reduced samples were studied and since the use of whole platelets, rather than mem-

TABLE I

THE RELEASE OF [^{14}C]SEROTONIN AND [^{3}H]ADENINE FROM WASHED PLATELETS BY PROTEASE TREATMENT IN THE PRESENCE AND ABSENCE OF RELEASE INHIBITORS

Labelled platelets were treated with proteases for 10 min at 37°C in the presence and absence of 1 mM prostaglandin E_1 and 1 mM acetylsalicylic acid, as described in Materials and Methods. The suspension was rapidly centrifuged and the supernatants analysed for the release of [^{14}C]serotonin and of [^{3}H]adenine as described in Materials and Methods.

Platelet treatment		Release inhibitors	[^{14}C] Serotonin release (%)	[^{3}H] Adenine release (%)
Substance	μg/ml			
Buffer	—	—	0	0
		+	0	0
Chymotrypsin	200	—	0	0
		+	0	0
Trypsin	50	—	71	13
		+	18	3
Pronase	50	—	44	11
		+	0	0
Papain	25	—	59	11
		+	0	1

brane preparations meant that at the maximum protein levels that could be successfully submitted to electrophoresis, the glycoproteins were diluted relative to total protein content. In the enzyme-treated samples, although glycoprotein III is relatively unchanged, the concentrations of glycoproteins

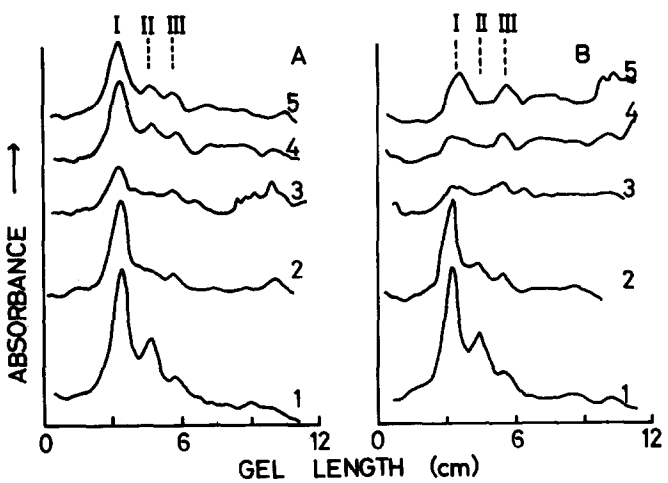

Fig. 1. Densitometer scans of material staining for carbohydrate with periodic acid-Schiff's reagent after gel electrophoresis of solubilised platelets which had been treated with proteases. Electrophoresis was performed on 5% polyacrylamide gels in the presence of 0.1% (w/v) sodium dodecyl sulphate. Platelets in the presence (A) and in the absence (B) of 1 mM prostaglandin E_1 and 1 mM acetylsalicylic acid were treated with 1, buffer; 2, chymotrypsin, 200 μg/ml; 3, trypsin, 50 μg/ml; 4, pronase, 50 μg/ml; 5, papain, 25 μg/ml, washed and subsequently solubilised in 3% (w/v) sodium dodecyl sulphate as described in Materials and Methods.

TABLE II

THE ABILITY OF PROTEASE-TREATED PLATELETS TO AGGREGATE IN RESPONSE TO RISTOCETIN AND TO BIND ^{3}H-LABELLED DB.BDB-IgG

Washed platelets were treated with proteases in the presence and absence of the release reaction inhibitors 1 mM prostaglandin E_1 and 1 mM acetylsalicylic acid as described in Materials and Methods. The binding of ^{3}H-labelled DB.BDB-IgG to these platelets and their agglutination response to ristocetin were measured as described in Materials and Methods.

Platelet treatment		Release inhibitors	Binding of ^{3}H-labelled DB.BDB-IgG (μg per $5 \cdot 10^8$ platelets)	Ristocetin-induced agglutination (%)
Substance	μg/ml			
Buffer	—	—	0.86	100
		+	0.82	100
Chymotrypsin	200	—	1.0	42
		+	0.85	47
Trypsin	50	—	1.12	0
		+	1.20	0
Pronase	50	—	0.93	0
		+	1.24	0
Papain	25	—	1.02	0
		+	1.11	0

I and II are decreased. This decrease was more marked in platelets treated with trypsin, pronase and papain, particularly in the absence of release inhibitors. That the decreased removal of glycoproteins in the presence of release inhibitors is not due to inhibition of protease activity by prostaglandin E_1 and acetylsalicylic acid was demonstrated by the failure of these substances to alter the caseinolytic activity of the proteases. Thus, as described by other authors [16,47,48], during the release reaction some carbohydrate-containing material with the same electrophoretic mobility as membrane glycoprotein I is liberated from the platelet.

The ristocetin-induced agglutination of platelets treated with proteases in both the presence and absence of release inhibitors was less than that observed in controls (Table II). Chymotrypsin treatment caused a 55% inhibition of platelet response while trypsin, pronase and papain abolished it completely. In contrast, the ability of platelets to bind ^{3}H-labelled DB.BDB-IgG was slightly enhanced. This enhancement of binding was less pronounced with chymotrypsin-treated platelets than with those treated with other enzymes in either the presence or the absence of release inhibitors.

The effect of ADP

Ristocetin-induced platelet agglutination was inhibited by low concentrations of ADP (Table III) and at 1 mM ADP was completely abolished. Binding of aggregated IgG remained unaltered even at high ADP concentrations.

The effect of ionophore A23187

Addition of A23187 to a platelet suspension induced a slight increase in light

TABLE III

EFFECT OF ADP ON BINDING OF ^{3}H-LABELLED DB.BDB-IgG TO PLATELETS AND ON THEIR RISTOCETIN-INDUCED AGGLUTINATION

Measurement of ^{3}H-labelled DB.BDB-IgG binding in platelet-rich plasma was performed as described in Materials and Methods. The platelet suspension was incubated with either ADP or 0.15 M NaCl for 2 min prior to the addition of ^{3}H-labelled DB.BDB-IgG. Ristocetin-induced agglutination was measured as described in Materials and Methods. The platelet suspension was platelet-rich plasma diluted with 0.14 M NaCl, 5.5 mM glucose, 5 mM EDTA, 10 mM Tris, pH 7.4. Either ADP or 0.15 M NaCl were preincubated with the suspension prior to the addition of ristocetin to a concentration of 0.9 mg/ml.

ADP (μM)	Binding of ^{3}H-labelled DB.BDB-IgG (μg per 5 · 10^{8} platelets)	Ristocetin-induced agglutination (%)
—	0.20 (0.19—0.21) (4)	100
1.25	not done	34
12.5	not done	3
125	0.22 (0.20—0.24) (4)	6

transmission, followed by a decrease, the magnitude of which was greater as the ionophore concentration increased (Fig. 2). These changes were roughly paralleled by the release of [^{14}C]serotonin, which was the same whether or not ristocetin was subsequently added. In the ristocetin control without ionophore no release was observed. Slight leakage of cytoplasmic [^{3}H]adenine nucleotides (12% at 3 μM ionophore) occurred but would not indicate major platelet lysis. The decrease in light transmission after ionophore addition was not due to ADP-mediated platelet aggregation since the experiments were performed in 4 mM EDTA, but appeared rather to be due to changes in the shape and size of the platelets; microscopic examination of these suspensions showed that the ionophore-treated platelets had lost their discoid shape and had many pseudopodia.

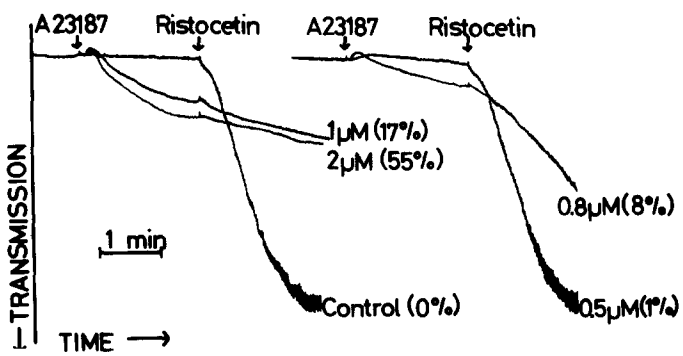

Fig. 2. Effect of ionophore A23187 on ristocetin-induced platelet agglutination. Platelet agglutination was measured by the change in light transmission of a suspension of platelets which had been labelled with [^{14}C]serotonin and [^{3}H]adenine as described in Materials and Methods. After the samples had been preincubated at 37°C for 2 min, ionophore A23187 was added in 1 μl ethanol so that the final concentrations were as indicated for each curve. Ethanol alone was added to the control. After a further 2 min, ristocetin was added to a final concentraiton of 0.75 mg/ml. The release of [^{14}C]serotonin at the end of the incubation, measured as described in Materials and Methods is shown in brackets for each concentration of ionophore A23187.

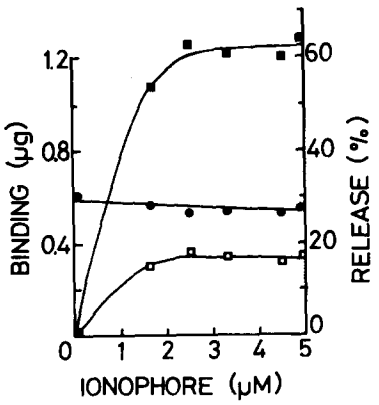

Fig. 3. Effect of ionophore A23187 on the ability of platelets to bind ^{3}H-labelled DB.BDB-IgG. A platelet suspension was incubated for 2 min at 37°C prior to the addition of ionophore A23187. After a further 3 min at 37°C ^{3}H-labelled DB.BDB-IgG was added and the binding, (●), expressed as μg bound per $5 \cdot 10^{8}$ platelets, during a further 2 min incubation was measured as described in Materials and Methods. The release reaction was measured in parallel samples containing platelets labelled with [^{14}C]serotonin and [^{3}H]adenine. These samples were treated in the same way except that ^{3}H-labelled DB.BDB-IgG was omitted and the supernatants of the suspensions after centrifugation were analysed for their ^{3}H and ^{14}C content. The release of [^{3}H]adenine (□), and [^{14}C]serotonin (■) thus represents that produced by ionophore alone.

The velocity of ristocetin-induced agglutination of ionophore-treated platelets decreased as the ionophore concentration was increased. Binding of ^{3}H-labelled DB.BDB-IgG was, however, not altered by the addition of ionophore at concentrations up to 5 μM (Fig. 3) although release of 63% [^{14}C]serotonin occurred between 1 and 5 μM A23187. As observed in the assay system used for the measurement of platelet agglutination the release of [^{3}H]-adenine nucleotides remained low.

Treatment of formaldehyde-treated platelets with proteases

Platelets in plasma were allowed to incorporate both [^{14}C]serotonin and [^{3}H]adenine and were then treated with formaldehyde. When these platelets

TABLE IV

THE EFFECT OF PROTEASE TREATMENT ON THE ABILITY OF FORMALDEHYDE-TREATED PLATELETS TO BIND ^{3}H-LABELLED DB.BDB-IgG AND TO AGGLUTINATE IN RESPONSE TO RISTOCETIN

The procedure was exactly that used in Table II except that platelets which had been treated with formaldehyde as described in Materials and Methods were used. Binding of ^{3}H-labelled DB.BDB-IgG was measured for 2 min at 37°C.

Platelet treatment		Binding of ^{3}H-labelled DB.BDB-IgG	Ristocetin-induced agglutination (%)
Substance	μg/ml	(μg per $5 \cdot 10^{8}$ platelets)	
Buffer	—	0.48	100
Chymotrypsin	200	0.49	100
Trypsin	50	0.54	38
Pronase	50	0.46	0
Papain	25	0.46	0

were treated with proteases no release of either isotope was observed in the absence of prostaglandin E_1 and acetylsalicylic acid. Treatment of these fixed platelets with pronase and papain abolished ristocetin-induced agglutination, trypsin treatment inhibited by 40% while chymotrypsin had no effect (Table IV). Binding of ^{3}H-labelled DB.BDB-IgG to protease-treated platelets did not differ significantly from the controls. Attempts were made to examine the glycoproteins of formaldehyde-treated platelets by gel electrophoresis; however, it was not possible to solubilize the platelets completely, even in the presence of higher concentrations of sodium dodecyl sulphate or with longer treatment at 100°C. After electrophoresis of the material that could be solubilised, almost no protein or carbohydrate was found to have entered the gel.

Discussion

These studies indicate clearly that the structures involved in the platelet receptors for aggregated IgG and ristocetin-von Willebrand factor are different and, in addition, provide some new information on the nature of these receptors.

Under conditions where the release reaction was largely inhibited it was shown that proteolytic treatment of platelets removed glycoproteins, thereby confirming the results of other authors [12,16,17,29—31]. Concomitantly the ability of the treated platelets to agglutinate to ristocetin-von Willebrand factor was lost and the binding of aggregated IgG was slightly enhanced.

Although Grant et al. [35] report that the release inhibitor prostaglandin E_1 slightly inhibits ristocetin-induced agglutination its presence in the experiments where release was inhibited cannot alone be responsible for the effects we observed since it was also present in the controls. The expression of the Fc receptor as measured by binding of ^{3}H-labelled DB.BDB-IgG is not affected by release inhibitors [11].

It also appeared unlikely that these effects could be due to subsequent action of residual protease, associated with the platelets, on ristocetin-von Willebrand factor or aggregated IgG in the assays, since, after incubation of platelets with the enzymes, inhibitors of protease action were added and the platelets thoroughly washed. Jenkins et al. [12] have also shown that only extremely low levels of chymotrypsin are associated with the platelets under the conditions used in these experiments.

Preincubation of platelets with ADP or with the ionophore A23187 also caused loss of the expression of the ristocetin-von Willebrand factor receptor. A23187 alters calcium permeability and appears thereby to induce the shape change and the release reaction [37—39]. Since in our experiments the inhibition of platelet response to ristocetin-von Willebrand factor after pretreatment with ionophore occurred only at ionophore concentrations where the release reaction, albeit only slight, occurred, it may be that released ADP was responsible for the effect rather than either a direct action of A23187 on the membrane structure or to membrane rearrangement accompanying the release reaction. On the basis of these results it could be possible that the protease treatment of platelets produced the apparent loss of the receptor for ristocetin-von Willebrand factor by means of changes in membrane configuration resulting

from the removal of substantial amounts of surface glycoprotein. However, since no enhancement of binding of aggregated IgG, as observed after protease treatment, was seen in the presence of even high concentrations of ADP or of ionophore it seems unlikely that such changes were responsible for all the observed effects on receptors after protease treatment of platelets.

These considerations were resolved by the use of platelets treated with formaldehyde. Since such platelets are "fixed", no shape change or release reaction occurred after protease treatment. Nevertheless, the ability of these platelets to agglutinate to ristocetin-von Willebrand factor was diminished by proteolysis. Although chymotrypsin did not alter the expression of the receptor, trypsin, pronase and papain did. The failure of chymotrypsin to modify the response of formaldehyde-treated platelets is not yet clarified. Thus the receptor(s) for ristocetin-von Willebrand factor would indeed appear to be material susceptible to proteolysis even after cross-linking of surface proteins with formaldehyde.

In contrast, no changes in the ability of formaldehyde-treated platelets to bind aggregated IgG were seen after protease treatment. The enhancement of binding seen after proteolysis of normal platelets was thus most probably due to either the removal of surface structures which hinder access to the Fc receptor or to the rearrangement of membrane constituents after proteolysis. Both of these effects should be minimised on platelets on which cross-linking by formaldehyde had occurred, since proteolysis would no longer necessarily lead to the removal or to the reorganisation of split products.

The results obtained here on the nature of the Fc receptor would appear compatible with those obtained for other cells. The activity of the Fc receptor of lymphocytes and of macrophages is enhanced by treatment with a wide range of proteases [10,49—52], but is destroyed by phospholipases A and C. Since protein-free liposomes show permeability changes when treated with aggregated IgG [53] it has been suggested that the Fc receptor may be lipid in nature. However, a glycoprotein with the properties of an Fc receptor has been isolated from murine lymphocytes [10]. Our studies show that the Fc receptor of platelets is not destroyed by protease treatment and is, therefore, most probably not one of the major surface glycoproteins. However, whether it consists of protein or glycoprotein more deeply buried in the membrane lipid layer or whether it is, in fact, lipid in nature awaits to be determined.

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