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Naturally occurring human IgG antibodies to intracellular and cytoskeletal components of human platelets

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SUMMARY

Immunoblotting of platelets that have been subjected to SDS–PAGE has revealed that sera from normal individuals contain IgG which binds to many platelet components. This binding was seen with autologous and heterologous platelets using serum of males and of nulliparous females who had not received blood transfusions. Although binding patterns of different sera were not identical, almost all sera caused IgG binding to platelet components of 87–90 kD, 140 kD (identified as vinculin) and 220–240 kD (tentatively identified as talin and actin-binding protein). Purified IgG showed the same binding pattern as whole serum and $F(ab')_2$ fragments retained their ability to bind to many components. The titre of IgG binding in serum was 1:50–1600 while that of alloantibodies to the PI^{A1} antigen was 1:3200. IgG binding components were not secreted when platelets were stimulated and were rarely associated with isolated membranes, but were located either in platelet cytoplasm or cytoskeletons. IgG binding was decreased by absorbing sera with lysed platelets or isolated cytoskeletons, but only slightly with intact platelets. Microaffinity purification of IgG which formed a major band on immunoblots showed that it was antibody with specificity for vinculin or its degradation products. These findings suggest that normal sera contain naturally occurring IgG antibodies with specificity for intracellular platelet antigens and that in some cases their titre approaches that of antibodies of pathological significance.

Keywords platelets antibodies natural autoantibodies cytoskeleton vinculin

INTRODUCTION

Naturally occurring antibodies to panels of defined protein Guilbert, Dighiero & Avrameas, 1982; Dighiero, Guilbert & Avrameas, 1982 and glycolipid (Kaise *et al.*, 1985) antigens have been found in sera of healthy humans and from unimmunized animals (Dighiero *et al.*, 1985; Ternynck & Avrameas, 1986; Underwood *et al.*, 1985a, b). Monoclonal paraproteins in patients with multiple myeloma also had antibody activity to autoantigens (Dighiero *et al.*, 1983). Most of these naturally occurring antibodies showed polyspecificity to a range of molecular and macromolecular antigens (Ternynck & Avrameas, 1986). Of 2306 hybrids examined by Underwood *et al.* (1985b), antigens to the monoclonal antibodies produced could be identified in 227. With the exception of one antibody, the antigens were associated with intracellular structures. Normal human sera contain antibodies to at least six components of red cell membranes (Lutz & Wipf, 1982) and there is suggestive evidence for the presence of antibodies to human platelets (Pfueller *et al.*, 1987; Steiner, 1985).

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In immunoblotting studies employing a sensitive immunogold technique we have observed that although IgG binding to specific platelet antigens can be seen with sera from patients with alloantibodies and quinine-dependent antibodies, there is also binding of IgG from normal sera to a number of platelet components (Pfueller *et al.*, 1988). Because this binding poses a problem in detecting specific binding of IgG in sera of patients with autoantibodies, we have investigated the nature and specificity of IgG binding in normal sera to human platelet antigens.

MATERIALS AND METHODS

Blood preparations

Blood was obtained from healthy normal consenting volunteers aged 20–45 years who had not received blood transfusions. This was obtained after incubating whole blood in glass tubes for 2 h at 37°C and 18 h at 2°C. For preparation of washed platelets, whole blood collected into one-tenth volume trisodium citrate was centrifuged at 800 *g* for 10 min. Platelet-rich plasma was removed and diluted in an equal volume of 0·01 M tris, 0·14 M NaCl, 0·027 M glucose, 0·005 M Na_2EDTA, pH 7·4 (TNGE) and sedimented by centrifugation at 2200 *g* for 10 min. Platelets were

washed by three cycles of resuspension in TNGE and centrifugation, and finally suspended in 0·14 M NaCl,0·005 M K_2EDTA, 0·01 M tris, pH 7·4, containing soybean trypsin inhibitor (11 g/l), 10 mM tosyl arginine methyl ester, 10 mM benzamidine, 10^7 U/l trasylol, sodium azide (1 g/l), 1 mM phenylmethyl sulphonyl fluoride and leupeptin (200 mg/l) (TNE: inhibitors).

Preparation of platelet subcellular fractions

Platelet membranes were prepared by sonication of washed platelets (1×10^9/ml) for 4×15 sec at 100 W (Labsonic 1510, B. Braun, Victoria, Australia). The pellet obtained by centrifugation at 19 000 *g* for 30 min as described (Käser-Glanzman *et al.*, 1977) was discarded and the membranes sedimented from the supernatant by centrifugation at 100 000 *g* for 1 h. The membrane fraction was washed once in TNE: inhibitors and resuspended in this solution. Platelet cytosol was the supernatant obtained after removal of the membrane fraction. Platelet-secreted proteins were obtained by incubating washed platelets suspended in TNGE buffer with thrombin (1 U/ml) for 10 min at 37°C. The suspension was diluted with an equal volume of ice-cold TNE:inhibitors and centrifuged at 100 000 *g* for 2 h. Only the upper two-thirds of the supernatant were used to study the secreted proteins to ensure that no membrane fragments or platelet microparticles were present. The pellet of the thrombin-treated platelets was suspended in the starting volume of TNE:inhibitors. Platelet cytoskeletons were prepared as described (Phillips, Jennings & Edwards, 1980) by adding an equal volume of 2% triton X-100, 0·01 M EGTA and 0·1 M tris, pH 7·4, to a suspension of 5×10^9 platelets/ml. After 5 min the cytoskeletons were removed by centrifugation at 11 000 *g* for 5 min and washed twice in a solution consisting of equal parts of the above extraction buffer and TNE:inhibitors and resuspended in this solution. Protein concentration was measured by the procedure of Lowry *et al.* (1951).

Preparation of IgG

IgG was obtained from serum by affinity chromatography on protein A–sepharose (Goding, 1976). $F(ab')_2$ fragments were prepared by digestion of IgG with immobilized pepsin (Pierce Chemical Co.) for 2 h at 37° at pH 4·5 and further passage through a protein A column. Fragments were free of intact IgG when examined on polyacrylamide gels.

Electrophoresis

SDS–PAGE was carried out as described (Laemmli, 1970) using a 5–15% gradient for 17 h at 22 V with 3% polyacrylamide as a sample loading gel. For most experiments, 6×10^8 platelets were loaded in a 125-mm well. In other experiments, individual 5-mm sample wells were used. One volume of platelet suspension was mixed with 10 volumes of a solution containing 0·003% bromophenol blue, 1·6% sodium dodecyl sulphate, 0·125 M sodium iodoacetamide, 6·5 M urea in 0·12 M tris HCl, pH 6·8, and incubated for 30 min at 37°C prior to loading. Molecular weight standards were either low (14·4–94 kD) or high (18·5–330 kD) molecular weight kits from Pharmacia, or pre-stained standards (BioRad, Richmond, CA) (17–130 kD) and IgG (Sigma, St Louis, MO) (160 kD) and fibrinogen (340 kD).

Electrophoresis was carried out for 17 h at 22 V.

Immunoblotting

Proteins were transferred to nitrocellulose (BioRad) as described (Towbin, Staehelin & Gordon, 1979). The portion of paper carrying the molecular weight standards was cut off and stained with colloidal gold (Moeremans, Daneels & de May, 1985) and the remainder was placed in blocking buffer containing skimmed milk powder (Diploma, Unilac, Australia) (50 g/l), 0·15 M NaCl, 0·075 M $CaCl_2$, sodium azide (1 g/l), 0·05% tween 20 (vol/vol) and 0·025 M tris-HCl, pH 8·1. In some experiments, to test different blocking agents, Hammarsten casein (Merck, Darmstadt, FRG) (2·5 g/l) was substituted for the skimmed milk powder in this buffer and the concentration of $CaCl_2$ increased to 0·15 M, or the buffer described by Nurden *et al.* (1985) containing 0·04% ficoll 400, 1·5% bovine serum albumin (BSA), 0·15 M NaCl, 0·15 $CaCl_2$, sodium azide (0·4 g/l), 0·1% nonidet P-40, 0·05 M tris-HCl, pH 8·1, was used. After 2 h incubation at 22°C, the nitrocellulose was washed twice for 15 min in blocking buffer. Nitrocellulose from transfers of platelets in 125 mm loading wells was cut into 4-mm strips and placed in 5 ml blocking buffer containing patient or normal serum for 16 h. The strips were then washed twice for 15 min in blocking buffer. IgG binding was visualized with gold-conjugated anti-human IgG by a modification of the sensitive procedure of Hsu (1984) as described by Pfueller *et al.* (1988). In some experiments gold conjugates of affinity isolated goat antibodies to human IgG (Sigma) or the IgG fraction of sheep serum containing anti-mouse immunoglobulins (Silenus, Victoria, Australia) were used. In experiments with $F(ab')_2$ fragments of IgG, the gold conjugate was prepared using affinity-purified antibody to human Fab fragments (Miles Scientific, Naperville, IL) because the anti-human IgG from Silenus had primarily Fc specificity and failed to detect binding to $F(ab')_2$ fragments. Pink bands were visualized after 1–3 h and maximum intensity was reached in about 18 h.

Preparation of platelet vinculin

A crude preparation of vinculin was prepared by the procedure of Feramisco & Burridge (1980) as modified by Puszkin *et al.* (1985). Briefly, washed human platelets were lysed by sonication and extracted in dilute tris–EGTA, followed by precipitations with $MgCl_2$ and $(NH)_4SO_4$. The crude preparation was subjected to preparative SDS–PAGE as described above. A portion of the gel was transferred electrophoretically to nitrocellulose and vinculin identified by incubation with a monoclonal antibody to vinculin (Sigma) followed by incubation with colloidal gold conjugated to the IgG fraction of antiserum to mouse IgG (Silenus). The remaining gel was stained with Coomassie blue and the band corresponding to vinculin as determined by the immunoblot was excised.

Micro-preparation of affinity-purified human naturally occurring anti-vinculin

Crude vinculin was applied to preparative SDS–PAGE and transferred to nitrocellulose as described above. Vinculin was identified on a portion of the nitrocellulose using monoclonal anti-vinculin. The strip of nitrocellulose corresponding to vinculin was excised from the unreacted portion of the blot and incubated with 4 ml of serum from an untransfused male donor in 20 ml of blocking buffer for 16 h at room temperature. The strip was then washed twice with 20 ml blocking buffer. Human naturally occurring anti-vinculin was then eluted by incubation

with 2 ml of 3 M KSCN for 5 min at 2°C. The eluate was diluted in 2 ml of distilled water and dialysed against five changes of 100 ml 0·05 mM disodium dihydrogen phosphate, 0·15 M NaCl, 0·01% sodium azide (w/v), 0·3% tween 20 (v/v), pH 7·2, and stored at −20°C until use.

Absorption of human serum with vinculin

A strip of nitrocellulose bearing vinculin was prepared as described above and incubated with 32 μl serum in 10 ml blocking buffer for 16 h at 22°C. A control strip from the 50 kD region of the immunoblot was incubated in the same way.

Measurement of antibody binding to intact platelets

Sera containing antibodies to Pl^{A1} were typed by the platelet immunofluorescence test (von dem Borne *et al.*, 1979) by the Red Cross Blood Bank. IgG binding to platelets was also measured by ELISA using microtitre plates coated with platelets as described (Pfueller *et al.*, 1987).

RESULTS

Detection of IgG binding from normal serum to platelet components

Immunoblotting of nitrocellulose strips bearing platelet components with serum from normal individuals (diluted 1:25) produced a variety of bands of different M_r (Fig. 1), the most common being those with M_r 230–240, 130–140, 80–90, 30–36 and 14–20 kD. When such strips were incubated with only blocking buffer prior to incubation with gold-conjugated anti-IgG, only non-specific bands corresponding to platelet-associated IgG were seen. When platelets of different individuals were used most bands obtained with a particular serum were similar, e.g. in Fig. 1 major bands of 240, 130, 90 and 40 kD occurred with both platelet donors. However, some bands appeared only with platelets from certain donors, e.g. in Fig. 1 serum B produced a number of low M_r bands with donor 2 and not with donor 1, while serum G produced heavy bands of 14 and 17 kD with donor 2 and not with donor 1. Band patterns were consistent when repeated with the same donor. When serum was incubated with blots prepared with autologous platelets (data not shown), IgG binding to many platelet components was also observed, with the M_r of the major bands being similar to those observed in the heterologous situation.

The intensity of Coomassie blue staining of proteins on the polyacrylamide gel was not related to the intensity of IgG binding on the immunoblot. In the experiment shown in Fig. 2, proteins identified as actin and myosin (Fox, 1985) did not bind IgG while strong binding was seen at the position of talin, previously termed P235 by Fox (1985), and some in the region of actin-binding protein (ABP). Strong IgG binding at 140 kD occurred in an area of very weak Coomassie blue staining.

Gold conjugates of IgG from another manufacturer gave similar patterns to those obtained with the usual anti-human IgG preparations, while conjugates of anti-mouse immunoglobulins gave only two extremely faint bands and BSA conjugated to gold gave no bands. IgG prepared from normal serum gave a similar pattern to that obtained with the whole serum. $F(ab')_2$ preparations from normal serum also bound to platelet antigens, although the intensity of many of the bands was decreased and some were missing. This binding was not a result of traces of residual intact IgG because it could not be detected when gold-conjugated anti-human IgG with specificity for the Fc rather than $F(ab')_2$ was used.

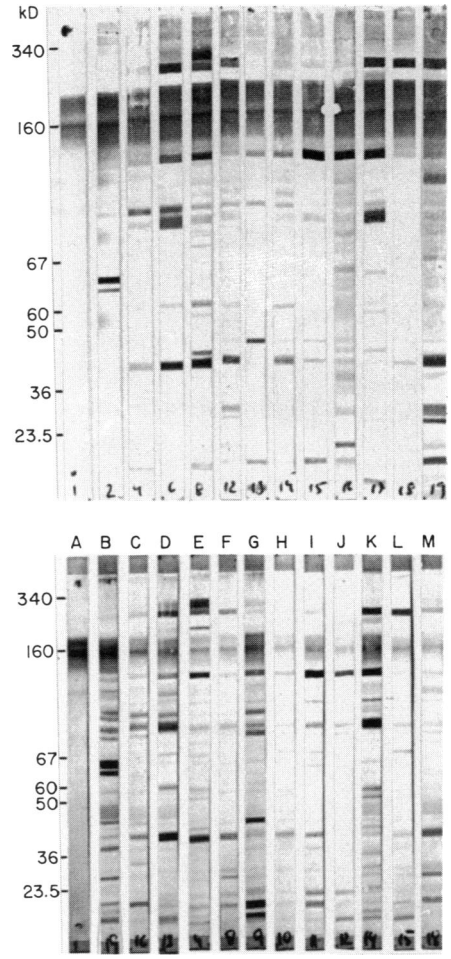

Fig. 1. Immunoblotting of normal platelets with normal sera. Platelets were subjected to electrophoresis on 5–15% polyacrylamide gels and transferred to nitrocellulose as described in Materials and Methods. The blot was cut into 4-mm wide strips and each was incubated with a 1:25 dilution of normal serum and IgG binding was visualized with sheep anti-human IgG conjugated with colloidal gold. Platelets from two normal donors (donor 1, top; donor 2, bottom) were incubated with buffer (A) or different sera (B–M).

The bands produced by normal serum were not significantly affected by the use of different buffers or different proteins in the blocking stage. Substitution of purified casein for the skimmed milk powder in the blocking buffer did not alter results, while substitution of 5% BSA or use of a buffer containing ficoll and BSA (Nurden *et al.*, 1985) allowed an unacceptably high background staining in the buffer control. To test the possibility that chemical modification of the platelet antigens during sample preparation may be responsible for the bands, platelet samples were boiled for 5 min or incubated at 37°C for 30 min in the presence or absence of urea. The band patterns were identical under all conditions.

The ability to detect IgG binding from normal sera was compared with that of serum containing anti-Pl^{A1} antibodies. In an ELISA using intact platelets, the anti-Pl^{A1} serum at a dilution of 1:1024 gave an absorbance reading of 0·73, while the upper limit of the normal range obtained with 18 normal sera at the

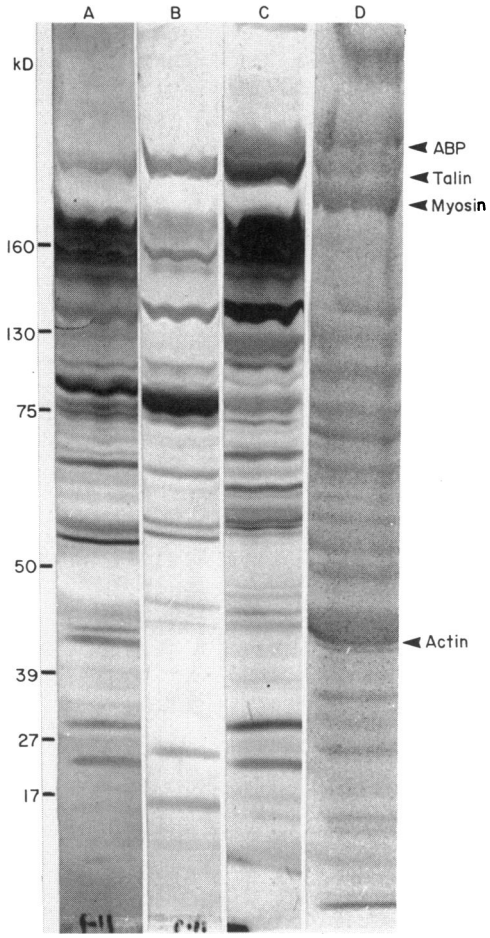

Fig. 2. Comparison of the protein staining of platelets with IgG binding from normal sera. Platelets were subjected to electrophoresis as described in Fig. 1. Platelet proteins were transferred to nitrocellulose and incubated with a 1:25 dilution of serum from three normal donors (A, B, C). IgG binding was visualized as described in Fig. 1. Known platelet proteins actin, myosin, talin (P235) and actin-binding protein (ABP) are indicated on a Coomassie blue stained portion of the gel (D).

same dilution was 0·095 (mean ± s.d.). On immunoblots, the 94-kD band with anti-Pl^{A1} serum was just visible at a dilution of 1:3200, some of those with normal sera were of a similar intensity at 1:800–1600, e.g. the 140 kD antigen in Fig. 3, while others were not detectable beyond 1:50, e.g. those at 100 and 40 kD. The intensity of all bands decreased and some faint ones disappeared when the incubation time of the nitrocellulose strips was decreased from 18 h to 1 or 2 h.

Subcellular location of IgG-binding components

IgG binding was similar with control or thrombin-treated platelets (Fig. 4). With the fraction secreted by thrombin only bands due to secretion of platelet-associated IgG (George *et al.*, 1985; Pfueller & David, 1986) were seen. Platelet membrane proteins bound little IgG while the greatest number of bands occurred with platelet cytosol and cytoskeletons. A similar distribution of IgG binding was seen with each of three normal sera tested.

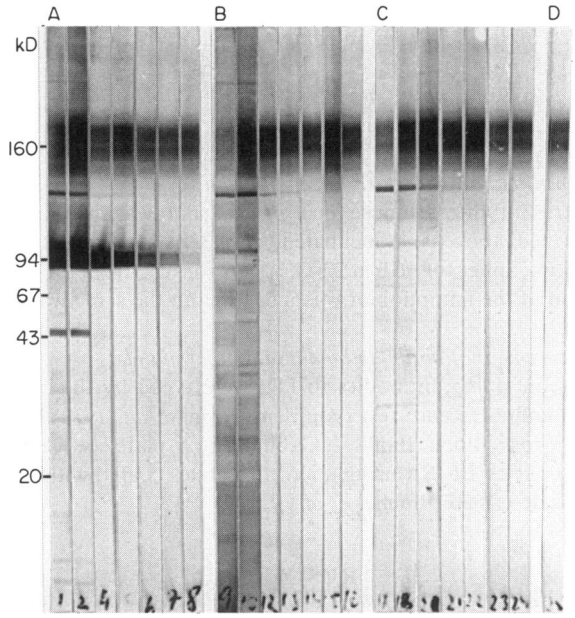

Fig. 3. Comparison of IgG binding from normal or anti-Pl^{A1} sera to platelet antigens. Blots of platelets were prepared as described in Fig. 1 and incubated with A, anti-Pl^{A1} serum; B and C, two different normal sera; D, blocking buffer alone. Each serum was diluted (from left) in blocking buffer 1:25, 1:50, 1:200, 1:400, 1:800, 1:1600 and 1:3200. IgG binding was visualized with colloidal gold conjugated to sheep anti-human IgG.

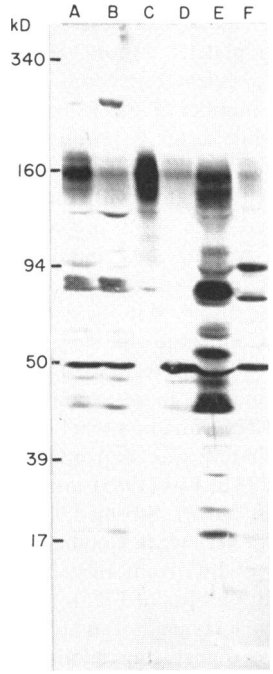

Fig. 4. Binding of IgG from normal serum to platelets and subcellular fractions. Blots were prepared from the following preparations as described in Fig. 1, but using individual sample wells: A, washed platelets; B, thrombin-treated platelets and C, from the secreted protein fraction; D, platelet membranes; E, cytosol fraction; or F, cytoskeletons.

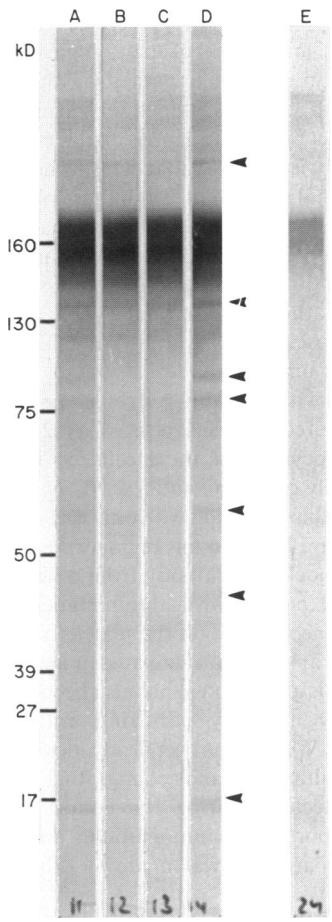

Fig. 5. Absorption of antiplatelet antibodies by platelet lysates and by cytoskeletons. Normal serum (100 μl) was used to resuspend A, 1×10^{10} intact platelets; B, cytoskeletons from 2×10^{10} platelets; C, 1×10^{10} sonicated platelets; or D, nothing, and incubated for 18 h at 2°C. The suspensions were then centrifuged for 15 min at 14 000 *g* at 2°C and the supernatants diluted 1:200 and incubated with platelet blots for 18 h as described in Fig. 1. IgG binding was visualised with colloidal gold conjugated to anti-human IgG as described. In E, binding of the anti-human IgG conjugate to the platelet blot incubated with buffer alone is shown. Bands showing decreased intensity after absorption of serum are indicated by arrows.

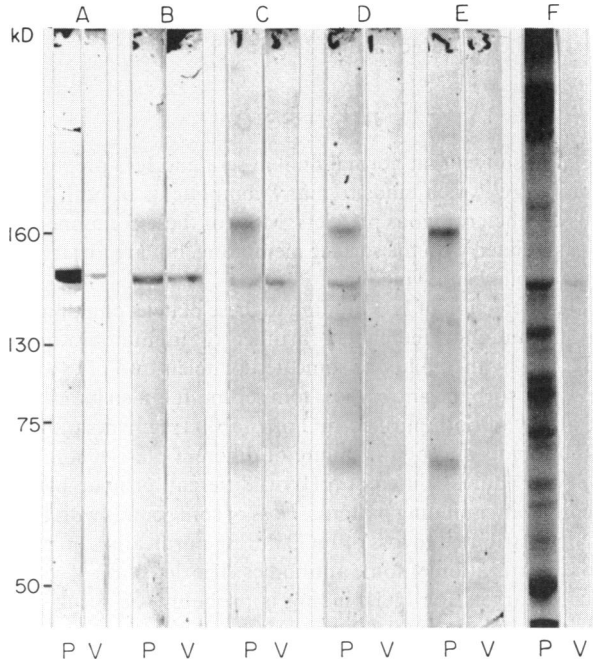

Fig. 6. Identification of a major autoantigen as vinculin. A portion of a strip of acrylamide gel containing purified vinculin (V), as described in Materials and Methods, or 5×10^7 washed platelets (P) were subjected to electrophoresis and transferred to nitrocellulose. They were incubated with mouse monoclonal anti-vinculin (A); human naturally occurring anti-vinculin (B); normal serum (C); normal serum absorbed with a strip from the non-vinculin region of a blot to which vinculin had been transferred (D); or normal serum absorbed with a strip of nitrocellulose containing purified vinculin (E), as described in Materials and Methods. IgG binding was visualized by incubation with gold conjugated to either sheep anti-mouse IgG (A) or sheep anti-human IgG (B–E). A portion of the gel stained with Coomassie blue is also shown (F).

Absorption of antibodies with platelets and cytoskeletons

Undiluted normal sera were pre-incubated with various platelet preparations prior to their dilution and incubation with platelet blots. Preliminary experiments showed that a dilution of 1:200 rather than 1:25 was required to make the amount of antibody limiting, thereby allowing a decrease in its amount to give proportional reduction in staining. Intact platelets caused the absorbtion of antibodies to few bands. A result typical of that obtained with six different sera is shown in Fig. 5, lane A, where only antibodies to 17 and 44 kD antigens have been absorbed. After absorption with isolated cytoskeletons or sonicated platelets, bands of 80 and 90 kD almost disappeared while those at 55, 140 and 240 kD showed minor changes (lanes B and C).

Identification of vinculin as a major antigen

Vinculin was purified from human platelets as described in Materials and Methods and used for the microaffinity purification of human naturally occurring anti-vinculin from the human serum used in Fig. 2, lane C, which gave a prominent 140 kD band. When purified vinculin and washed platelets were subjected to electrophoresis and examined on immunoblots with mouse monoclonal anti-vinculin, strong reactivity was seen at 140 kD. In the platelet preparation, weak staining at 135, 110 and 74 kD was also seen (Fig. 6, lane A). These bands were identical to those obtained when similar immunoblots were reacted with human naturally occurring anti-vinculin (Fig. 6, lane B) indicating that the bands of M_r lower than 140 kD were the result of binding of monoclonal mouse antibody and the naturally occurring human antibody to degradation products of vinculin rather than of multi-specificity of the antibodies to unrelated proteins. When blots were examined with the serum from which the human naturally occurring anti-vinculin had been prepared, in addition to these bands, platelet-associated IgG at 160 kD and some fainter bands were seen with the washed platelet preparation (Fig. 6, lane C). This serum was used at a higher dilution than in Fig. 2 so that reductions in the amount of antibody would give a clearly visible decrease in staining. After absorption of normal serum with purified vinculin, the 140 kD band obtained with both preparations decreased markedly (Fig. 2, lane E). Control absorption with nitrocellulose not containing vinculin did not alter the band pattern (Fig. 6, lane D). The 140 kD band corresponding to

vinculin is seen to be prominent with the sera in Fig. 2 and with most of the sera used in Figs 1, 3 and 5.

DISCUSSION

We have shown that normal human sera and IgG preparations contain IgG which binds to many components of both autologous and heterologous platelets. Most of these components are not associated with the surface membrane, but are intracellular, a major one being vinculin.

The detection of IgG binding to these platelet components is specific and not merely an artifact of immunoblotting, as indicated by the ability of sonicated platelets and of cytoskeletons to absorb the antibodies from serum. It is also indicated by the ability of both purified IgG or $F(ab')_2$ fragments to bind to immunoblots and by the failure of different platelet solubilization procedures, different pH or different protein blocking agents to alter binding patterns. Loss of some bands with $F(ab')_2$ may be attributed to loss of IgG3 during purification on protein A or denaturation of some antibodies. The detection of so many platelet targets for IgG binding is probably the result of the sensitive immunogold blotting system, together with long incubation times giving saturation binding. Specific antibody binding is further supported by the unique patterns produced by different sera as shown in Fig. 1. Furthermore, the ability of affinity purified human naturally occurring anti-vinculin to bind only to proteins recognized by a monoclonal antibody to vinculin suggests that IgG binding to vinculin in washed platelets is a result of specific recognition of epitopes rather than non-specific binding.

The dilutions at which IgG binding to some platelet components was detected approached that found with antibodies to the Pl^{A1} antigen. In particular, the band corresponding to anti-vinculin in Fig. 3 was detectable at just double the concentration required to visualize anti-Pl^{A1}. However, binding of IgG to other proteins could only be visualized at about 60-fold higher concentrations. Measurement of anti-platelet antibodies by ELISA of intact platelets shows markedly higher IgG binding with anti-Pl^{A1} sera than with normal sera (Pfueller *et al.*, 1987). However, a very low 'background' IgG binding was detectable even though the assay uses intact platelets, on which intracellular antigens are not exposed. Studies with naturally occurring monoclonal antibodies from normal non-immunized mice suggest that their affinities are comparable to those of antibodies obtained after specific immunization (Ternynck & Avrameas, 1986).

All sera used were from normal, non-transfused males or nulliparous non-transfused females, all with normal levels of platelets, and therefore the IgG binding we have observed appears to be the result of naturally occurring antibodies. Their predilection towards intracellular antigens supports the findings of Underwood *et al.* (1985b) with naturally occurring mouse antibodies obtained in monoclonal form. It is unlikely that our failure to detect membrane associated antigens was a result of an inability of such antigens to bind to nitrocellulose because the Pl^{A1} antigen, on membrane glycoprotein IIIa (McMillan *et al.*, 1980), and quinine- and quinidine-dependent antigens on other major surface glycoproteins, Ib, IIb, and IX have also been detected by this procedure (Pfueller *et al.*, 1988).

The presence of naturally occurring antibodies may pose a problem in the identification of target antigens in autoimmune thrombocytopenia where their presence in patient sera may be confused with antibodies of pathological significance. For example, IgG in sera from patients with thrombocytopenia associated with systemic lupus erythematosus have been reported to react with several intracellular components (Kaplan *et al.*, 1987). It is unlikely that densitometric subtraction of profiles of normal serum blots from those obtained with patient sera, as suggested for kidney microsomal antibody in liver disease (Kyriatsoulis *et al.*, 1987), would make identification of pathological antibodies more certain unless the amount and/or affinity of the pathological antibodies were more than four-fold higher than those of the naturally occurring antibodies as we have shown for anti-Pl^{A1}.

In common with senescent red cells, stored lymphocytes and neutrophils, stored platelets possess a 62-kD antigen which mediates phagocytosis of these cells by macrophages via a specific naturally occurring antibody (Kay, 1981). We found a strong band in this M_r region with only one serum (Fig. 1, serum B), although faint bands in this region were seen with most sera studied. A monoclonal antibody from a heterohybrid cell line derived from a patient with autoimmune thrombocytopenia reacted with a neoantigen on the platelet surface glycoprotein IIIa which only appeared on activated or aged platelets (Nugent *et al.*, 1987). This glycoprotein has also been postulated to act as a receptor for naturally occurring antibodies (Steiner & Luscher, 1986). We have not yet firmly identified antigens other than vinculin, although bands comigrating with ABP and talin have been detected. During the course of our studies, a preliminary report of immunoglobulin binding from normal sera to platelet antigens was made by others (Reid, Basta & Shulman, 1987).

Most of the naturally occurring antibodies we have observed appear to be directed to antigens which are not exposed on resting platelets, and it is therefore unlikely that they affect normal platelet function. However, thrombin treatment of platelets is known to make cytoskeleton proteins accessible to rabbit antibodies to actin, α-actinin and vinculin (Jenkins, Maimon & Puszkin, 1984). Thus, activated platelets, e.g. those in a thrombus, may have these intracellular antigens exposed to the extracellular milieu, and could thereby become targets for naturally occurring antibodies for example to vinculin, which we have identified here, and be removed by the cells of the reticuloendothelial system as suggested by Grabar (1975).

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