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Identification of Fc and F(ab')$_2$ IgG receptors on human platelets

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ABSTRACT **The human platelet receptors for normal, nonimmune IgG and its F(ab')$_2$ and Fc fragments were studied by the use of a cleavable, bifunctional, photoactivable, ^{125}I-labeled cross-linking agent. Derivatization of the ligands with *N*-[4-(*p*-azido-*m*-[^{125}I]iodophenylazo)benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester (Denny–Jaffe reagent) reduced their binding to platelets by ≥20%. Cleavage of the azo linkage of the Denny–Jaffe reagent, which splits the molecule so that its ^{125}I-labeled portion becomes associated with the receptor half of the cross-linked ligand-receptor complex, was utilized to directly identify receptors for the various immunoglobulin ligands. Specificity of the binding reaction could be demonstrated by suppressing the iodination of the receptors with excess nonderivatized ligand. Two principal IgG-related receptors could be identified by high-resolution NaDodSO$_4$/PAGE and subsequent analysis of the electrophoretically transferred peptides to nitrocellulose filters for localization of radioactivity and immunological characterization. Intact monomeric IgG and F(ab')$_2$ fragments derived from it appeared to have the glycoprotein IIIa as the major receptor, whereas Fc fragments bound predominantly to a peptide of M_r ≈200,000 (M_r, ≈50,000 under rigorous reducing conditions).**

The presence of IgG receptors on human platelets has been shown (1, 2). Experiments in this laboratory (2) implied that F(ab')$_2$ bound to glycoprotein IIIa, whereas experiments by Rosenfeld *et al.* (1) showed Fc bound to a 40-kDa peptide. In the latter studies indirect evidence for the binding of the Fc fragment was obtained with monoclonal antibodies, whereas in the former studies IgG derivatives were directly cross-linked to human platelets followed by identification of the cross-linked receptor-ligand complex. We now report the use of the ^{125}I-labeled, bifunctional, cleavable cross-linking agent *N*-[4-(*p*-azido-*m*-[^{125}I]iodophenylazo)benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester (Denny–Jaffe reagent, New England Nuclear) (3, 4) to characterize the binding sites for Fc and F(ab')$_2$ normal human IgG fragments and compare these binding results with those obtained using intact IgG (2).

The techniques that have been successful with small molecular weight hormones (5) are difficult to apply to large ligands. Therefore, to directly demonstrate binding, IgG and IgG fragments were conjugated with a cross-linking agent. The success of such studies depends on the derivatized ligand behaving like the native molecule.

The present studies show similar glycoprotein IIIa binding activity for derivatized and normal IgG or for its F(ab')$_2$ fragments, whereas normal or derivatized Fc fragments were bound to a high molecular weight platelet protein (M_r, ≈200,000). Binding specificity of derivatized IgG and its fragments was verified because nonderivatized IgG, F(ab')$_2$, or Fc inhibited binding of labeled compounds. All of our present studies were performed exclusively with monomeric IgG.

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METHODS AND MATERIALS

Preparation of Platelets. Platelets were isolated from acid citrate/dextrose (pH 5.1) (ACD; ref. 20) anticoagulated blood (6). After careful removal of contaminating erythrocytes, platelets were sedimented, and the resulting pellets were resuspended in 0.015 M sodium phosphate (pH 7.2) containing 0.14 M NaCl (PBS) and 15 vol% ACD (final pH, pH 6.0) (6). To optimize dissociation of platelet-bound IgG, the platelets were allowed to sit at room temperature for 30 min (7). The platelets were again sedimented by centrifugation and then resuspended in PBS. The final cell concentration was adjusted to 2×10^9 platelets per ml.

Derivatization of IgG and Its Fragments F(ab')$_2$ and Fc with the Denny–Jaffe Reagent. Human IgG from a commercial source and IgG isolated from freshly collected, normal human plasma (2) was dissolved in 0.2 M sodium borate (pH 8.5), pooled to a final concentration of 20 mg/ml, and centrifuged at 100,000 × *g* for 60 min. The content of the upper half of the centrifugation tube was removed. To document the monomeric nature of the IgG, one portion of this fraction was further analyzed in an analytical ultracentrifuge. A single peak sedimenting with an $s_{20,w}$ of 7.1 was observed. Purity of the IgG was ascertained by immunoelectrophoresis and by NaDodSO$_4$/PAGE under conditions of complete reduction and alkylation. Protein concentrations were determined by the method of Lowry *et al.* (8) using bovine serum albumin as standard. The IgG and F(ab')$_2$ preparations had no anti-P1^{A1} activity (P1^{A1}, a platelet specific antigen) by binding studies comparing P1^{A1}-positive and P1^{A1}-negative platelets.

Monomeric IgG F(ab')$_2$ [obtained either commercially or prepared by treating IgG with immobilized pepsin and separating the resulting F(ab')$_2$ fragments on an immobilized protein A column (9)] or Fc fragments dissolved in 0.2 M sodium borate (pH 8.5) were treated with Denny–Jaffe reagent. Between 0.6 and 1 mg of protein was added to 145–150 μCi of ^{125}I-labeled cross-linking agent and incubated for 75 min at room temperature in complete darkness (1 Ci = 37 GBq). The reaction was terminated by the addition of 0.1 ml of 0.5 M lysine (pH 8.5). The derivatized IgGs or IgG fragments were passed over a preequilibrated Sephadex G-25 column (preequilibration, 1% bovine serum albumin in PBS followed by extensive washing with PBS until the A_{280} was ≥0.005). Protein peaks were collected, and the protein concentration of the peaks was again determined.

Cross-Linking of ^{125}I-Labeled, Derivatized IgG, F(ab')$_2$, and Fc to Platelet Membranes. The incubation mixtures for the cross-linking experiments contained 4×10^{10} platelets, 0.7 μM derivatized IgG, 1 μM derivatized F(ab')$_2$ or 2 μM derivatized Fc, and bovine serum albumin at a final concentration of 0.5 g/dl. As a control for specific binding, nonderivatized IgGs or IgG fragments were added in 10-fold excess over the ligand conjugated with cross-linking agents. The reaction mixtures were incubated for 20 min at room temperature. The platelets were then sedimented by centrifugation and resuspended in PBS containing bovine serum albumin 0.5 g/dl and 15 vol% ACD. Two brief washing steps followed. Up to this point, all procedures were performed in

complete darkness. The platelet suspensions were then illuminated for 15 min with a UVL-21 lamp (Ultraviolet Products, San Gabriel, CA) delivering 420 $\mu W/cm^2$ of 365-nm light at a distance of 15 cm. The platelet suspension in an open glass cuvette was placed under a 1.5-cm deep Pyrex Petri dish and gently mixed twice during the course of the illumination. To cleave the cross-linked material, 0.1 vol of 2 M dithionite was added three times at 15-min intervals. After centrifugation, the platelets were washed two more times before being solubilized in 0.0625 M Tris, 1 mM EDTA, 1% $NaDodSO_4$, and 2% (vol/vol) glycerol containing 40 mM dithiothreitol, and the protein concentration was adjusted to 1 mg/ml. The solubilized samples were immediately boiled for 3 min. If not immediately used for electrophoresis, the samples were stored at −80°C.

Completeness of dithionite cleavage was measured by comparing the radioactivity in the area where cross-linked material migrates in the gel before and after dithionite treatment. Approximately 80% of the cross-linked material was dissociated by dithionite.

PAGE. High-resolution gradient gels were prepared according to Clemetson *et al.* (10) and electrophoresed at a constant current of 10 mA per gel. The gels were calibrated with the following prestained standard proteins: myosin (M_r, 200,000), β-galactosidase (M_r, 116,000), phosphorylase *b* (M_r, 95,000), glutamate dehydrogenase (M_r, 55,000), ovalbumin (M_r, 43,000), lactate dehydrogenase (M_r, 36,000), carbonic anhydrase (M_r, 29,000), lactoglobulin (M_r, 18,400), and cytochrome *c* (M_r, 12,400). The gels were either stained by the silver-staining method (11) or electrophoretically transferred onto nitrocellulose filters according to Towbin *et al.* (12). The efficiency of the blotting procedure was periodically checked. High molecular weight proteins (M_r, >100,000) showed a moderate decrease of transfer to nitrocellulose. The transfer efficiency varied between 80% and 90%. To evaluate the distribution of radioactivity, the nitrocellulose blots were cut into 2-mm wide sections, and radioactivity was measured in a γ counter (counting efficiency for ^{125}I was ≥80%). Blotted peptide bands were identified with IgG antibodies having specificity for both heavy and light chains and with antibodies to lithium diiodosalicylate-extracted platelet membrane proteins. Procedural details of these methods have been published (2).

Measurement of Platelet-Associated IgG. The amount of normal or derivatized IgG, $F(ab')_2$, and Fc fragments bound to platelets was measured by the use of fluorescein isothiocyanate-conjugated goat anti-human IgG serum. Details of the method have been described (7).

Materials. Platelets were isolated from freshly donated blood of volunteers. *N*-[4-(*p*-azido-*m*-[^{125}I]iodophenylazo)-benzoyl]-3-aminopropyl-*N'*-oxysuccinimide ester was purchased from New England Nuclear. The specific activity was 2200 Ci/mmol. Human IgG was obtained from Sigma; $F(ab')_2$ and Fc fragments of human IgG were obtained from Calbiochem. All immunoglobulins were pure as judged by immunoelectrophoresis and $NaDodSO_4$/PAGE. Prestained standard proteins were obtained from Diversified Biotech (Newton Center, MA); protein A-gold and enhancer kit were from Bio-Rad. Glucose oxidase–anti-glucose oxidase complexes were a product of Accurate Chemical & Scientific (Westbury, NY).

RESULTS

The Denny–Jaffe reagent was easily attached to human IgG or its $F(ab')_2$ and Fc fragments. The reaction, performed in complete darkness, yielded IgG with an average of 1 mol of cross-linking agent bound per 650 mol of IgG. $F(ab')_2$ had a molar ratio of 1:1706, and Fc had a ratio of 1:2270 (mol of Denny–Jaffe reagent attached per mol of immunoglobulin fragment, respectively). The capacity of the intact immunoglobulin and its fragments derivatized with cross-linking agent to bind to normal platelets was tested directly by determining the ^{125}I radioactivity associated with platelets (Table 1) or indirectly with fluorophore-conjugated anti-human IgG (13) (Table 2). Compared to pooled normal IgG, $F(ab')_2$, and Fc fragments, the derivatized ligands had moderately decreased ability to bind to platelets. The decrease did not exceed 20% with any of the ligands. Because direct and indirect measurements of the binding of derivatized IgG and its $F(ab')_2$ and Fc fragments to platelets gave comparable results, the conjugation procedure appears to do minimal damage to the IgG ligands. Derivatized and nonderivatized immunoglobulins seem to have similar binding characteristics.

The ligands conjugated with cross-linking agent were analyzed by $NaDodSO_4$/PAGE (Figs. 1–3). Under conditions of reduction and alkylation, the derivatized immunoglobulin and immunoglobulin fragments yielded the expected pattern of polypeptides. $F(ab')_2$ was incompletely reduced under the experimental conditions used, separating primarily into Fab fragments. Only with very high concentrations of DL-dithiothreitol or 2-mercaptoethanol could complete reduction of Fab be achieved. Each major polypeptide band was still able to react with a polyclonal anti-IgG antibody after electrophoretic transfer to nitrocellulose. In addition to the immunoblot bands associated with the major radioactive peaks, several other bands reacted with anti-IgG antibody. These most likely represent incompletely reduced IgG fragments. The pattern of radioactivity showed fairly equal distribution of the cross-linking agent among the various reduced portions of the three immunoglobulin ligands.

Cross-linking of intact IgG to normal human platelets produced two major peaks of radioactivity that corresponded to those seen with reduced IgG conjugated with Denny–Jaffe reagent (Fig. 4). Other than a relatively small number of counts remaining at or near the interface of stacking and running gel, several peaks of radioactivity were found in the M_r 60,000–115,000 range. Labeling was inhibited by excess unlabeled normal IgG in only the M_r 110,000–115,000 peak (Fig. 4*B*). The antiserum against lithium diiodosalicylate-extracted platelet membrane proteins bound to this peptide. Studies with $F(ab')_2$ produced similar results (Fig. 5). Labeled Fc fragments, however, bound to only a single high molecular weight peptide with an estimated M_r ≈200,000 (Fig. 6), and excess normal Fc fragments inhibited labeling of this peptide (Fig. 6*B*). Under more rigorous reducing conditions (2% 2-mercaptoethanol), this high molecular weight peptide dissociated into monomers of M_r ≈50,000. With both IgG fragments the major peaks of radioactivity were those expected of free ligand.

Table 1. Binding of derivatized IgG and IgG fragments to platelets

Denny–Jaffe reagent, μM	Protein, fg per platelet		
	IgG	$F(ab')_2$	Fc
0.2	0.36, 0.30	0.24, 0.25	0.08, 0.07
1.0	1.26, 1.35	1.1, 1.0	0.5, 0.47

Platelets freed of prebound immunoglobulin were incubated with IgG or IgG fragments that had been conjugated with ^{125}I-labeled cross-linking agent. The platelets were reacted in complete darkness with the Denny–Jaffe reagent at the indicated concentrations. After removing loosely adherent ligand in the dark (7), the radioactivity associated with the platelets was measured. From the specific activities of IgG and its fragments, absolute amounts bound per platelet were calculated. The results of two separate experiments are reported.

Table 2. Binding of IgG and IgG fragments to platelets

	Protein, fg per platelet		
	IgG	$F(ab')_2$	Fc
Derivatized	1.20, 1.34	1.58, 1.52	1.42, 1.50
Nonderivatized	1.50, 1.48	1.66, 1.57	1.81, 1.75

Platelets freed of prebound immunoglobulin were incubated with normal or derivatized IgG or its $F(ab')_2$ and Fc fragments at a concentration of 1 μM. The amount of immunoglobulin or its fragments bound was determined with fluorophore-conjugated rabbit anti-human IgG antibody. The resulting fluorescence associated with platelets was measured by fluorospectrophotometer. Details of this technique have been described (7). The data of two binding experiments are listed. The left-hand value of each pair shows the results obtained with platelets from a PI^{A1}-positive donor, whereas the right-hand value is data obtained with platelets of a PI^{A1}-negative donor. The values shown in this table indicate the amount of fluorophore-conjugated rabbit anti-human IgG antibody bound per platelet.

DISCUSSION

The identification of the receptors for large molecular weight ligands by the use of cross-linking agents poses special problems not shared by small-sized ligands. Two-dimensional electrophoresis does not always allow clear separation of the cross-linked receptor-ligand complexes from other complexes or aggregates that migrate to the interface between stacking and running gel. Therefore, to establish clear identification of the receptor, an additional purification and separation step of the IgG receptor-ligand complex is needed. The use of the ^{125}I-labeled, bifunctional, cross-linking agent used in this study provides an alternate solution to this problem. As cleavage of the azo linkage connecting the two functional ends of the cross-linking agent does not involve reduction of a disulfide bridge, sulfhydryl-reducing agents can be used in the first dimension of the $NaDodSO_4$/PAGE and thus obviate the need for a second-dimension gel. In addition, the transfer of the ^{125}I label from the ligand to the receptor simplifies recognition of the receptor and enhances

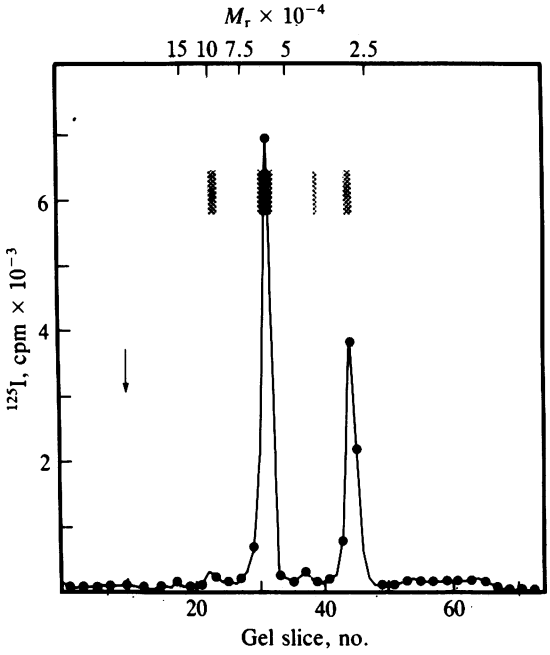

FIG. 1. Separation of IgG derivatized with ^{125}I-labeled cross-linking agent on $NaDodSO_4$/polyacrylamide gradient gels. Protein was reduced with 40 mM dithiothreitol prior to electrophoresis. After the electrophoretic separation, the peptides were immediately blotted onto nitrocellulose. One aliquot of each sample was used for the immunologic identification of the peptides, whereas the other was divided into sequential segments for the determination of radioactivity. Cross-hatched areas represent peptides that reacted with anti-human IgG having both heavy and light chain specificity. To avoid overcrowding with experimental data, some points have been omitted from the graph. However, the solid line traces the exact distribution of counts along the full length of the gel. The arrow indicates the start of the running gel.

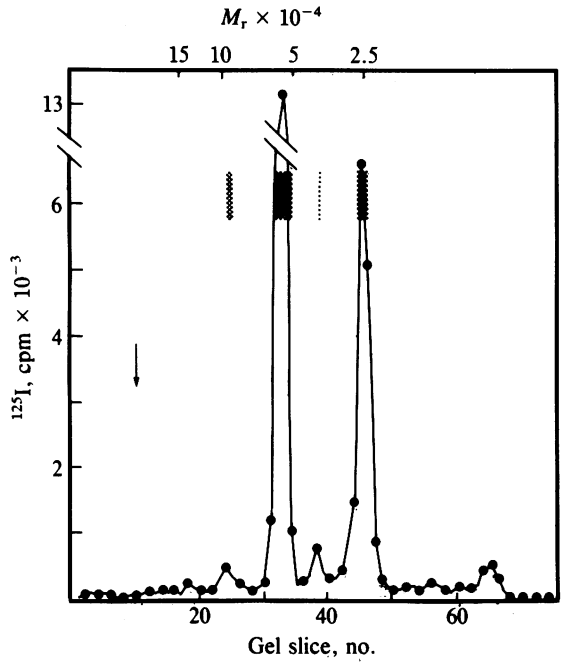

FIG. 2. Distribution on $NaDodSO_4$/polyacrylamide gradient gels of radioactivity associated with $F(ab')_2$ IgG fragments derivatized with ^{125}I-labeled cross-linking agent. Conditions were identical to those given in Fig. 1. Cross-hatched areas represent bands that reacted with antibody to human IgG having both heavy and light chain specificity. Presentation of the data as in Fig. 1. The arrow indicates the interface between stacking and running gel.

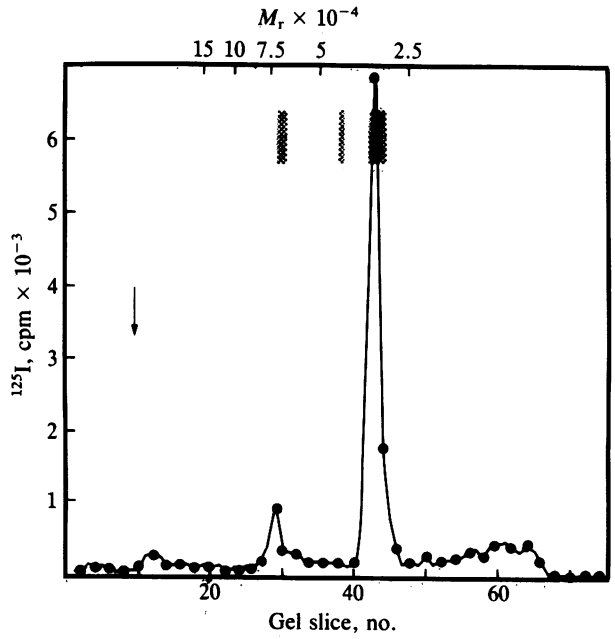

FIG. 3. Separation of Fc IgG fragments derivatized with ^{125}I-labeled cross-linking agent on $NaDodSO_4$/polyacrylamide gradient gels. Conditions of analysis and presentation of data are as described in Fig. 1. The arrow indicates the top of the running gel. The cross-hatched areas represent bands that reacted with anti-human IgG of both heavy and light chain specificity.

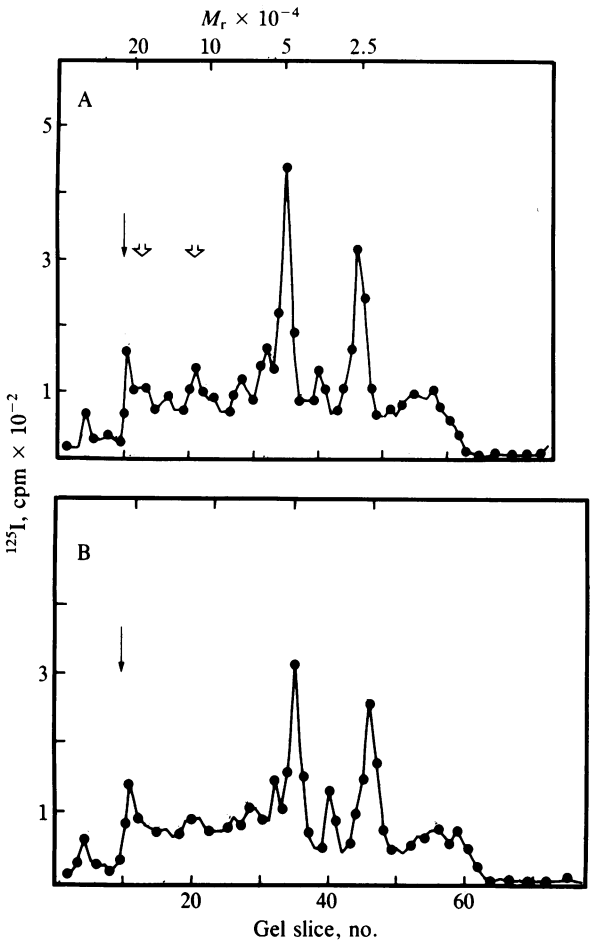

FIG. 4. Distribution of ^{125}I-labeled proteins derived from platelets that were incubated under standard conditions with 0.7 μM IgG derivatized with cross-linking agent (*A*). The cross-linked complexes that formed during photoactivation were cleaved with dithionite before application to the gels. The two wide arrows in *A* indicate the position of bands that showed specific suppression of ^{125}I incorporated upon exposure to a 10-fold excess (7 μM) of nonderivatized IgG (*B*). Other conditions of experimental analysis and presentation of data were as described in Fig. 1. The thin arrow represents the interface of stacking and running gel.

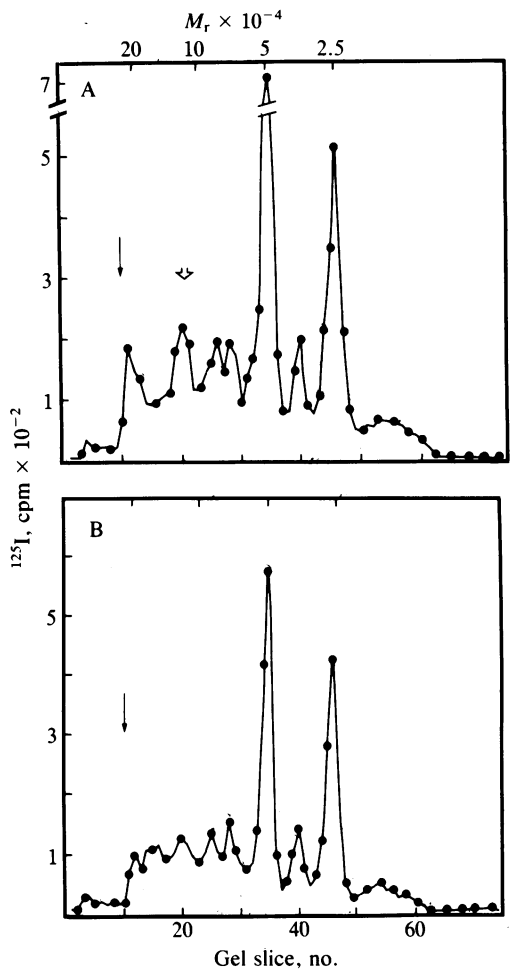

FIG. 5. Distribution of radioactivity from platelets that were incubated under standard conditions with 1 μM F(ab')$_2$ derivatized with ^{125}I-labeled cross-linking agent (*A*). Cross-linked complexes that formed during photoactivation were cleaved by treatment with dithionite prior to electrophoresis. The wide arrow in *A* indicates the peptide whose ^{125}I labeling could be suppressed by addition of an excess (10 μM) of underivatized F(ab')$_2$. The profile of radioactivity of platelets exposed to a 10-fold excess of normal F(ab')$_2$ is shown in *B*. Conditions of experimental analysis and presentation of data are described in Fig. 1. The thin arrows show the position of the interface between running and stacking gel.

reliability of identification. Use of this cross-linking agent, however, does not resolve the identification problem completely as the individual radioactive peaks represent either unbound ligands and their components produced by complete reduction or receptors labeled by ligand cross-linking and subsequent cross-link cleavage. Therefore, potential ambiguity remains. We determined the identity of the individual radioactive peaks by the immunoblot technique using selected antibodies whose presence could be shown at high sensitivity with gold-conjugated protein A or with a glucose oxidase–antiglucose oxidase system. With these techniques and by comparing the electrophoretic patterns of radioactive peptides of the original derivatized ligands with the distribution of peptides derived from platelets cross-linked with derivatized IgG or IgG fragments, we were able to determine the identity of virtually all the major radioactive peaks. In this study we again observed that glycoprotein IIIa was the receptor for intact IgG confirming the result obtained with a different cross-linking agent (2).

It was interesting to note that F(ab')$_2$ fragments not only were bound to platelets but appeared to bind to the same receptor glycoprotein IIIa. Fc fragments, on the other hand, were bound to a high molecular weight protein, clearly different from glycoprotein IIIa. The estimated molecular weight (M_r, ≈200,000 or M_r, ≈50,000 under rigorous reducing conditions) of this protein is very similar to that described for the nonspecific IgG binding site in platelet immunoblots (14) and is also similar to that described by Cheng and Hawiger (15) using platelet lysate fractionation on IgG affinity columns. We are unable to explain the discrepancy between our results and the reported (1) identification of the Fc receptor as a M_r 40,000 peptide. Although we did observe a radioactive M_r 40,000 peptide after dithionite cleavage, we were unable to identify this peptide as Fc receptor. Its labeling was not inhibited by excess normal, nonderivatized Fc fragments in the incubation mixture. Furthermore, pure Fc fragments conjugated with Denny–Jaffe reagent consistently yielded a radioactive peak in the M_r 40,000 area of the gels.

The present experiments were performed with monomeric IgG. Monomeric IgG has been shown to have relatively few binding sites on platelets (16), distinctly different from aggregated IgG (17). It has been shown that human platelets contain a platelet-specific IgG that was isolated by cell chromatography (18). We speculate that the platelet-specific, "nonimmune" IgG represents an antibody against a senescence antigen on platelets. The IgG associated with platelets

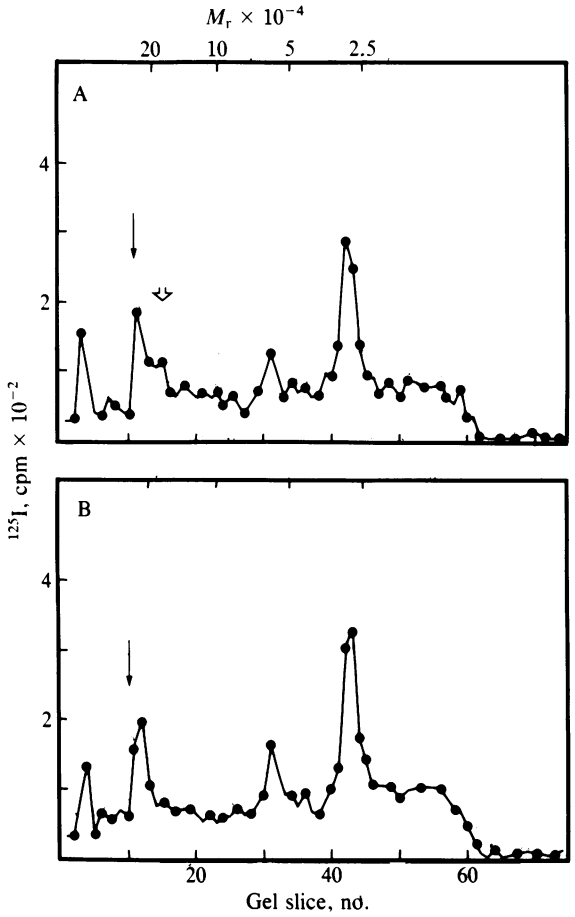

FIG. 6. Distribution of ^{125}I-labeled proteins derived from platelets that were incubated under standard conditions with 2 μM Fc derivatized with ^{125}I-labeled cross-linking agent (*A*). Cross-linked complexes formed during photoactivation were cleaved by treatment with dithionite before electrophoresis. The effect of a 10-fold excess (20 μM) of nonderivatized Fc on the profile of radioactivity of platelets is shown in *B*. The wide arrow in *A* indicates the positions of the peptide that showed specific suppression of its radioactivity by a large excess of normal, nonderivatized Fc fragments. Conditions of experimental analysis and presentation of data have been described in Fig. 1. The thin arrow indicates the top of the running gel.

increases with the age of the cells, and the binding of platelets to macrophages is related to the amount of IgG carried on the platelet surface (19). At this time we have no firm data to suggest the nature of the senescence antigen; however, an alteration in the conformation of glycoprotein IIIa, possibly resulting from repeated agonistic stimulation during the life span of the platelet yet insufficient to cause removal of the platelet through irreversible aggregation, may be an explanation.

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