Membrane-bound Hemagglutinin Mediates Antibody and Complement-dependent Lysis of Influenza Virus-treated Human Platelets in Autologous Serum

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Abstract. Influenza A virus-treated human platelets were lyzed in autologous serum. Lysis required the presence of antibody and occurred predominantly through activation of the classical complement pathway. Binding of the virus followed by its elution at 37°C resulted in a dose-dependent desialation of the cells with a maximal release of 45% of total platelet sialic acid. In contrast, platelets that had been treated with *Vibrio cholerae* neuraminidase and from which 55% of total sialic acid had been removed were not lyzed in autologous serum and did not bind C3 as shown in binding assays using radiolabeled monoclonal anti-C3 antibody. Thus, the immune-mediated lysis of virus-treated platelets in autologous serum did not involve neoantigens expressed by desialated cells. To assess the effect of viruses on the platelet surface, treated platelets were incubated with galactose oxidase and sodium [^{3}H]borohydride prior to separation and analysis of the labeled glycoproteins by SDS-PAGE. Viral treatment resulted in a desialation of each of the surface glycoproteins. At the same time, a labeled component of M_r 72,000 (nonreduced) and M_r 55,000 (reduced) was observed that was not present when *V. cholerae*-desialated platelets were examined in the same way. Immunoblotting experiments performed using antiwhole virus and anti-hemagglutinin antibodies demonstrated this component to be viral hemagglutinin. Involvement of membrane-bound hemagglutinin in antibody and in complement-mediated lysis of virus-treated platelets in autologous serum was supported by the increased lytic activity of a postvaccinal serum containing an elevated titer of complement fixing anti-hemagglutinin antibodies. Binding of a viral protein to the platelet surface provides a model for immune thrombocytopenias occurring during acute viral infections at the time of the specific immune response.

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Introduction

Thrombocytopenia may accompany or follow the onset of a variety of viral diseases. These include infections with ortho and paramyxoviruses, such as influenza (1, 2), Newcastle (3), measles (4), or rubella (5) and other viruses such as chicken pox (6), Dengue (7), and cytomegalovirus (8). Thrombocytopenia, which may be associated with a shortened survival of platelets after transfusion (9), usually occurs after maximal viremia, at a time when a specific immune response has developed. Thus, accelerated clearance of platelets could follow adsorption of immune complexes to platelet Fc receptors; alternatively, antibodies could interact with neoantigens expressed on altered platelet membranes after their contact with virus or with platelet-associated viral antigens in a mechanism analogous to that recently demonstrated for immune-mediated thrombocytopenias in malarial infections (10).

Influenza viruses bind to specific receptors on cell membranes and elute from the cells through cleavage of *N*-acetyl neuraminic acid residues by viral neuraminidase (11, 12). The accelerated clearance of platelets in vivo following their interaction with influenza virus in vitro has been related to virus-induced desialation of the cells (1, 3, 13). As it has been suggested for erythrocytes (14), thrombocytopenia in this model could result from the binding of desialated platelets to galactose receptors on cells of the reticulo-endothelial system; other mechanisms for enhanced clearance of desialated cells could involve C3b deposition following activation of the alternative complement pathway (15, 16) and/or lysis following antibody-dependent activation of the classical pathway (17, 18). In the present study, we provide evidence to show that incubation of

human platelets with suspensions of influenza virus, but not with bacterial neuraminidase, results in complement and antibody-mediated lysis of platelets in autologous serum. The crucial event was the interaction of immunoglobulins with viral hemagglutinin present on the platelet membrane. Such a mechanism could provide a major pathway for platelet clearance in viral infections at the time of the specific immune response or in hosts that had been preimmunized with crossreactive viral antigens.

Methods

Buffers. Veronal-buffered saline (VBS)[1], VBS containing 0.15 mM calcium and 0.5 mM magnesium (VBS^{++}), VBS containing 0.04 M EDTA (VBS-EDTA), VBS containing 2 mM magnesium and 8 mM EGTA (VBS-Mg EGTA), VBS containing 0.1% gelatin (GVB), GVB containing 2 mM magnesium and 8 mM EGTA (GVB-Mg EGTA) and GVB containing 0.04 M EDTA (GVB-EDTA), were prepared as described (19, 20).

Washed platelets. In a typical experiment, blood (100 ml) was collected from a healthy adult donor into two 50 ml tubes each containing 7.5 ml acid-citrate-dextrose anticoagulant (National Institutes of Health formula A) and 50 units of heparin (Choay, Paris, France). Platelet-rich plasma was prepared by centrifugation at 120 *g* for 15 min at 30°C. The platelets were sedimented by centrifugation at 1,000 *g* for 15 min at 30°C, resuspended in 10 ml of Tyrode's buffer, pH 7.4, containing 0.35% wt/vol bovine serum albumin (BSA; Tyrode-albumin), apyrase (purified, 0.03 μg/ml, kindly provided by Dr. J.-P. Cazenave, Strasbourg, France) and 100 units of heparin, and incubated in this buffer for 10 min at 37°C. After addition of prostacyclin (PGI_2) (10^{-7} M), the platelets were sedimented at 700 *g* for 6 min at 30°C. The platelets were resuspended and washed once more as above except for the fact that heparin was omitted from the buffer. Finally, the platelets were resuspended in Tyrode-albumin buffer containing apyrase and used within 4 h. When needed, ^{51}Cr- or [^{3}H]serotonin labeling of the platelets was performed in the first step of the washing procedure during platelet isolation by incubating 10^{10} platelets with 200 μCi of ^{51}Cr or 30 μCi of [^{3}H]serotonin (Amersham, Arlington Heights, IL). Lactate dehydrogenase activity was determined using a commercial kit (Boehringer Mannheim Diagnostics, Inc., Houston, TX).

Treatment of platelets with purified influenza virus preparations. Human influenza A Hong Kong/1/68 (H3N2) or influenza A Bangkok (H3N2) virus was grown in allantoic cavities of 11-d-old embryonated chicken eggs for 48 h. Infective allantoic fluids were clarified by centrifugation at 5,000 *g* for 15 min at 4°C. The supernatant was centrifuged at 65,000 *g* for 15 min at 4°C and the pelleted virus was further purified by absorption to and elution from formalinized chicken erythrocytes (21, 22). Purified viruses were resuspended in 0.025 M Tris HCl, 0.138 M NaCl, 5 mM dextrose, 0.9 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5 mM KCl, 0.2 mM Na_2HPO_4, pH 6.9, and used immediately. Viral concentration was measured using an hemagglutination test and expressed as hemagglutinating units (HAU) per milliliter (11).

For viral treatment of platelets, 1×10^9 washed platelets were incubated with purified influenza virus (800–20,000 HAU) in 5 ml Tyrode-albumin buffer containing apyrase for 15 min at 4°C. Incubation was performed at low temperature to allow the viruses to bind to the cell membrane without inducing platelet agglutination or granule release. When platelets were washed after their incubation with virus, no hemagglutinating activity could be detected in the supernatant, indicating that in the experimental conditions that were used, all viral particles became bound to the platelets at 4°C. The platelet suspensions were then incubated in Tyrode-albumin buffer containing apyrase for 60 min at 37°C to allow neuraminidase activity and viral elution. The platelets were sedimented by centrifugation at 700 *g* for 5 min at 30°C and washed once prior to their resuspension and incubation for another 60 min at 37°C in the same buffer. Viral particles were effectively eluted from the treated platelets during the incubations at 37°C since all the virus was recovered as hemagglutinating activity in the washes and since no residual viral particles were seen on examination of the surface of treated platelets by scanning electron microscopy (Fig. 1). Conventional transmission electron microscopy of 2.5% glutaraldehyde-fixed platelets demonstrated that treated platelets were disc-shaped with a normal granule content. In some cases, one or two ingested viral particles were seen in vesicles in intact cells. Virus-treated platelets were used immediately. Control platelets were prepared under the same conditions but in the absence of virus.

Treatment of platelets with Vibrio cholerae neuraminidase. For treatment with bacterial neuraminidase, 6×10^8 washed platelets in 1.0 ml of 0.05 M Na acetate, 0.135 M NaCl, 4 mM $CaCl_2$, pH 6.5, were incubated for 45 min at 37°C with an equal volume of buffer containing 0.04–1.0 IU of *V. cholerae* neuraminidase (<0.0002 um/mg protease; Boehringwerke AG, Marburg-lahn, West Germany). The treated platelets were sedimented at 700 *g* for 5 min at 30°C and resuspended in Tyrode-albumin buffer. For determination of sialic acid associated with membranes of untreated platelets, platelets that had been treated with viral particles, or those treated with bacterial neuraminidase, 6×10^8 cells were hydrolyzed in 0.1 N H_2SO_4 at 80°C for 60 min and free sialic acid was assayed by the thiobarbituric acid method (23).

Serum. Serum was obtained from the platelet donors and used immediately or stored at −70°C. For one donor, serum was obtained before and after immunization with influenza vaccine (Mutagrip, Institut Pasteur, Paris). Postvaccinal serum from this donor was absorbed with purified virus (21) by incubating 1.0 ml of serum with an equal volume of VBS^{++} containing 150,000 HAU for 4 h at 2°C. Virus particles were pelleted by centrifugation at 65,000 *g* for 12 h at 4°C. Serum from a child with primary agammaglobulinemia was obtained before and after treatment with gammaglobulins and was a kind gift from Dr. A. Fischer (Hôpital des Enfants Malades, Paris). The total complement hemolytic activity (CH50) of the serum before and after treatment was normal. Anti-hemagglutinin antibodies in serum were measured using an inhibition of hemagglutination test (HAI) (11).

Anti-viral antibodies. Viral hemagglutinin (HA) was purified from influenza A virus 3QB (H3N2) Hong Kong variant after treating the purified virus with bromelain as described (24, 25). Anti-whole virus

1. *Abbreviations used in this paper:* CH50, total hemolytic complement; HA, hemagglutinin from influenza virus; HAI, inhibition of hemagglutination test; HAU, hemagglutinating units; GVB, veronal-buffered saline containing 0.1% gelatin; GVB-EDTA, GVB containing 0.04 M EDTA; GVB-Mg EGTA, GVB containing 2 mM magnesium and 8 mM EGTA; pl-v, human platelets that have been treated with influenza virus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VBS, veronal-buffered saline; VBS^{++}, VBS containing 0.5 mM magnesium and 0.15 mM calcium; VBS-EDTA, VBS containing 0.04 M EDTA; VBS-Mg EGTA, VBS containing 2 mM magnesium and 8 mM EGTA.

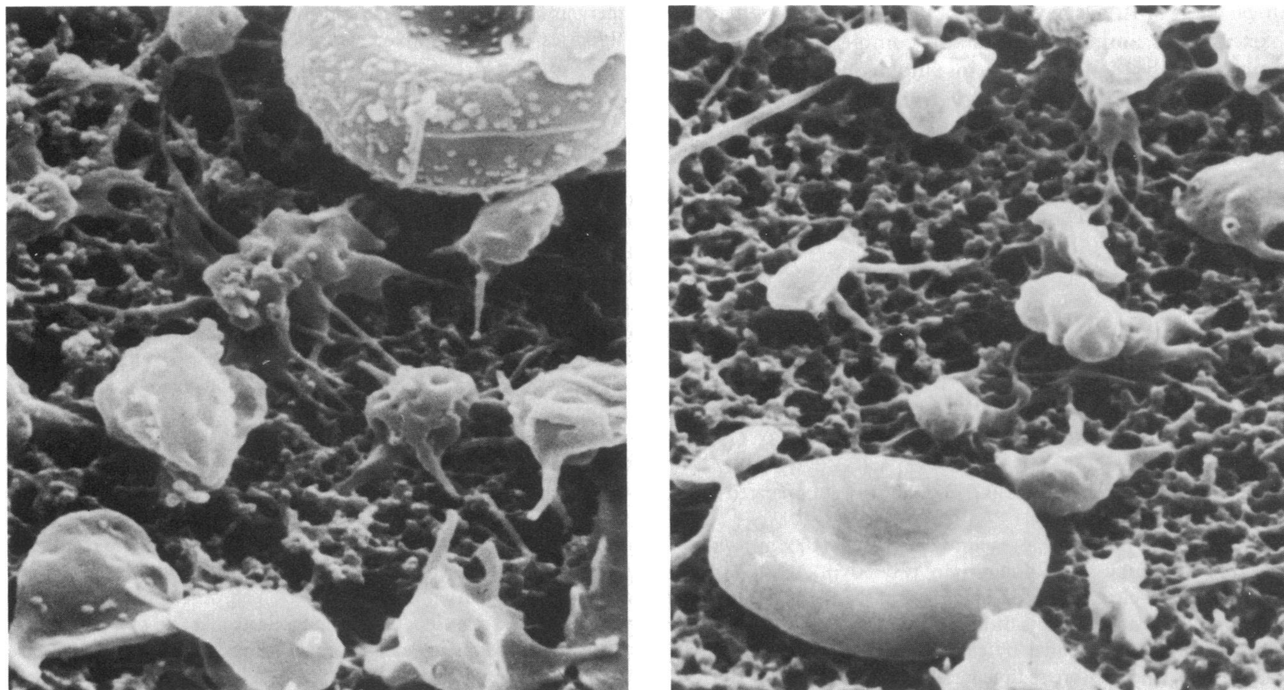

Figure 1. Scanning electron microscopy of human platelets to which influenza A virus particles have bound during incubation at 4°C (left). After viral elution at 37°C, no residual virus particles are seen on the treated cells (right). A field including an erythrocyte has been chosen to better demonstrate the presence and absence of virus.

antibodies were obtained in rabbits (21). Anti-HA antibodies were obtained first by injecting 100 μg of purified HA in complete Freund's adjuvant into the footpads of a Fauve de Bourgogne rabbit and by boosting the animal after 1 and 3 wk with 200 μg of protein in CFA injected intramuscularly and after 6 wk by injecting intravenously 100 μg of antigen. The rabbit was bled 5 d after the last injection. The titer of anti-viral and anti-HA antibodies in sera from immunized animals was determined using HAI and an enzyme-linked immunosorbent assay (21).

Complement assays. CH50 (26), C3 (27), and B (28) serum hemolytic activities were measured as described. C2 hemolytic activity was assessed using cellular intermediates bearing guinea pig C1 and human C4 (29).

C3 uptake studies. In vitro C3 uptake onto platelets was quantitated using mouse monoclonal anti-human C3 IgG antibody that had been purified from ascitic fluid (Bethesda Research Laboratories, Gaithersburg, Md.) by affinity chromatography on protein A sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). The antibody was radiolabeled with ^{125}I (Amersham Corp.) to a specific activity of 350,000 cpm/μg by means of Iodo-Gen (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Aliquots of 10^9 platelets were incubated in 1 ml of a 1:3 dilution of autologous serum in VBS^{++}, GVB-Mg EGTA, or GVB-EDTA[2] for 60 min at 37°C. After addition of 3 ml of ice-cold GVB-EDTA, the platelets were pelleted, washed once in GVB-EDTA, and incubated in 0.3 ml of the same buffer containing radiolabeled antibody (900,000 cpm) for 30 min at 30°C. Duplicate 75 μl samples of the reaction mixture were then centrifuged through a mixture of oil for 1 min at 8,000 *g* (30). The tubes were cut and radioactivity associated with the platelets was determined in an LKB 1275 gamma counter (LKB Instruments, Inc.). Specific C3 deposition on platelets was calculated by first substracting the number of ^{125}I-labeled monoclonal anti-C3 antibody molecules that bound to untreated and to neuraminidase or virus-treated platelets incubated in serum diluted in GVB-EDTA from that bound to platelets incubated in serum diluted in VBS^{++} or GVB-Mg EGTA[2]. These values were then used in substracting the number of antibody molecules bound to untreated platelets from that which bound to treated platelets.

Surface labeling and analysis of platelet glycoproteins by SDS-polyacrylamide gel electrophoresis (PAGE). Washed suspensions of platelets at 1×10^9/ml were either incubated with purified virus (20,000 HAU) as described in the previous section or *V. cholerae* neuraminidase (1 IU/ml) during 1 h at 37°C. Appropriate controls were performed in the absence of added virus particles or *V. cholerae* neuraminidase. ^{3}H-labeling of the surface glycoproteins of treated platelets was performed according to the procedure of Nurden et al. (31). In brief, suspensions of 10^9 platelets in 1 ml phosphate-buffered saline (PBS), pH 7.4, were first incubated with 10 U/ml of galactose oxidase (Kabi Diagnostics, Stockholm, Sweden) for 5 min at 37°C. After sedimentation and resuspension in 1 ml PBS, the platelets were incubated with 0.5 mCi sodium [^{3}H]borohydride (16 Ci/mmol; Commissariat à l'Energie Atomique, Saclay, France) for 15 min at room temperature. After washing,

2. Dilution of the serum in VBS^{++} allows complement activation by both the classical and the alternative complement pathways. Dilution in Mg EGTA excludes classical pathway activation.

the ^{3}H-labeled platelets were resuspended at 2 × 10^9 platelets/ml in Tris–HCl, 0.15 M NaCl, 3 mM EDTA, pH 7.0, and solubilized by heating at 100°C for 5 min in the presence of 2% (wt/vol) SDS and 5 mM *N*-ethylmaleimide. When performed, disulfides were reduced by incubating SDS-solubilized samples at 37°C for 1 h in the presence of 5% 2-mercaptoethanol. All materials for SDS-PAGE were of electrophoresis purity grade and were obtained from Bio-Rad Laboratories (Richmond, CA). These included the high and low molecular weight standards used for molecular weight determination by SDS-PAGE. Samples (100 μg protein) were analyzed by electrophoresis on 7–12% gradient acrylamide slab gels (31, 32). Proteins were detected by Coomassie blue staining and ^{3}H-labeled glycoproteins by fluorography using EN^{3}HANCE (New England Nuclear, Boston, MA) (31). The gels were dried onto filter paper and exposed to Kodak X-Omat MA film (Kodak-Pathé, Paris, France) for up to 3 wk at −70°C.

Identification of platelet-bound virus antigens using Western blots. Virus-treated platelets were prepared as described above and solubilized directly with SDS without prior ^{3}H-labeling. Samples (50 μg protein) were electrophoresed with and without disulfide reduction on 7–12% gradient acrylamide slab gels. A small aliquot (10 μl) of a 0.05 mg/ml solution of pyronin Y was added to each sample to act as a marker for the front migration after the electrophoresis. Transfer of the separated polypeptides to nitrocellulose membranes (Bio-Rad Laboratories) was performed according to the method of Towbin et al. (33). Prior to contact with the antibody, individual strips were routinely soaked for 3 h in 50 mM Tris-HCl, pH 8.1, containing 0.15 M $CaCl_2$, 0.04% (wt/vol) Ficoll, 1.5% (wt/vol) BSA, 0.04% (wt/vol) NaN_3, and 0.1% (wt/vol) Nonidet P-40. Further steps were as detailed by Bennett et al. (34) with slight modifications. Strips were incubated for 2 h with a 1:10 dilution of a serum containing either (*a*) anti-whole virus antibodies or (*b*) anti-HA antibodies; or (*c*) with control nonimmune rabbit sera. After multiple washings, the membranes were incubated overnight with 1 ml (300,000 cpm) ^{125}I-Protein A (10 μCi/μg protein; New England Nuclear). The strips were again exhaustively washed, dried, and the radiolabeled bands detected by autoradiography.

Results

Antibody-dependent lysis of virus-treated human platelets in autologous serum through activation of the classical complement pathway. 4 × 10^8 washed ^{51}Cr-labeled untreated platelets, or platelets that had been treated with 20,000 viral HAU (pl-v) were incubated in 1 ml of a 1:3 dilution of autologous serum in VBS^{++} at 37°C with constant agitation. At times 0, 60, and 210 min, duplicate 100-μl aliquots of the reaction mixture were taken, centrifuged for 1 min at 8,000 *g*, and the supernatant assessed for ^{51}Cr release and for residual hemolytic complement activity. In a typical experiment, incubation of virus-treated platelets in autologous serum resulted in 11 and 47% ^{51}Cr release and in 35 and 58% decrease in CH50 at 60 and 210 min, respectively, whereas incubation of untreated platelets yielded 2.4 and 5.3% ^{51}Cr release and 13 and 17% decrease in CH50 (Fig. 2). Release of lactatedehydrogenase paralleled that of ^{51}Cr (not illustrated). Autologous platelets were lyzed to a similar extent within a ±5% range in serum from five other healthy donors. Incubation of pl-v in autologous serum diluted in VBS^{++} was associated with a significant decrease in C2, C3, and B hemolytic activities (Table I). A small decrease in C3 and B but not in C2 hemolytic activities was observed when pl-v were incubated in autologous serum diluted in GVB-Mg EGTA; this limited amount of alternative pathway activation did not result in >4% specific ^{51}Cr release. No complement consumption occurred when pl-v were incubated in autologous serum diluted in GVB-EDTA and when untreated platelets were incubated in autologous serum diluted in either buffer. If EDTA (40 mM final) was added to the reaction mixture after 60 min of incubation of pl-v in autologous serum diluted in VBS^{++}, no increase in ^{51}Cr release

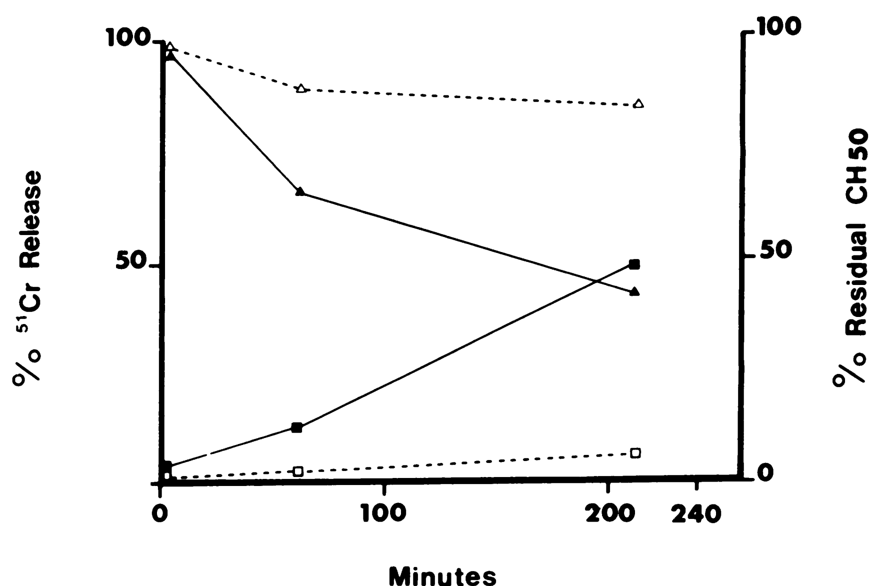

Figure 2. Kinetics of ^{51}Cr-release (squares) and of CH50 (triangles) during incubation of 4 × 10^8 influenza virus-treated (closed symbols and full lines) or untreated (open symbols and dotted lines) human platelets in 1 ml of a 1:3 dilution of autologous serum in VBS^{++}.

Table I. Complement Activation following Incubation of Untreated (pl) or pl-v in Autologous Serum (AS) Diluted in VBS^{++}, GVB-Mg ETA, and GVB-EDTA

	Percent residual hemolytic activity*			
	CH50	C3	C2	B
pl in AS-VBS^{++}	86.0	73.0	92.8	78.0
pl in AS-GVB-Mg EGTA	86.0	90.5	107.5	100.0
pl in AS-GVB-EDTA	100.0	89.0	150.0	92.0
pl-v in AS-VBS^{++}	18.5	46.0	4.0	51.0
pl-v in AS-GVB-Mg EGTA	67.0	71.1	130.0	65.0
pl-v in AS-GVB-EDTA	100.0	89.0	148.0	109.0

* Results are expressed as the mean of three experiments.

occurred upon further incubation of the platelets for 120 min at 37°C. Thus, lysis of pl-v occurs in autologous serum through predominant activation of the classical complement pathway. When pl-v were incubated in serum from a hypogammaglobulinemic patient for up to 90 min at 37°C, no specific ^{51}Cr release and no complement consumption occurred. However, incubation of pl-v in serum that was obtained from the same patient after replacement therapy with gamma-globulins resulted in 25% lysis of the cells and 94% complement consumption. Although the experiment was performed in an allogeneic system, it indicated that antibody was required for classical pathway-dependent lysis of influenza virus-treated platelets in human serum.

Absence of complement activation on Vibrio cholerae neuraminidase-treated platelets. To examine whether lysis of pl-v was mediated by natural antibodies recognizing antigenic sites exposed on the platelets after virus-induced desialation of the cells, platelets were treated with bacterial neuraminidase under conditions where the extent of desialation was comparable with that caused by the virus. Treatment of washed platelets with increasing amounts of influenza virus or *V. cholerae* neuraminidase resulted in a dose-dependent desialation of the platelets until a plateau of 45 and 55% desialation was reached for cells that had been treated with more than 6,500 HAU or 0.6 IU of neuraminidase/10^9 platelets, respectively (Fig. 3). Significantly, incubation in autologous serum diluted in VBS^{++} of platelets from which 50% of the membrane-associated sialic acid had been removed using *V. cholerae* neuraminidase did not result in lysis of the cells nor in complement consumption. The number of C3 molecules that specifically bound to *V. cholerae* neuraminidase-treated platelets following their incubation in autologous serum diluted in VBS^{++} or in GVB-Mg EGTA was quantitated and compared with that which bound to virus-treated platelets under the same experimental conditions. No C3 specifically bound to neuraminidase-treated platelets, whereas 5,000 and 1,600 C3 molecules (per platelet) bound to pl-v following their incubation for 60 min in autologous serum diluted in VBS^{++} and Mg EGTA, respectively (Fig. 4). The number of C3 molecules which bound to pl-v

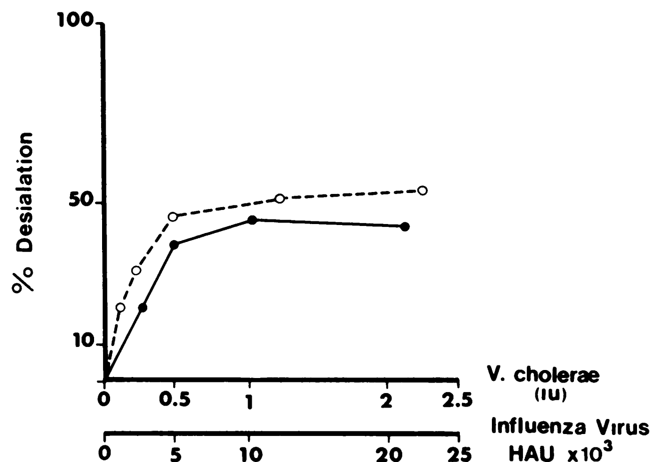

Figure 3. Relative amount of membrane-associated sialic acid removed from 10^9 human platelets following treatment with increasing amounts of neuraminidase from *V. cholerae* (--- ○ ---) and of influenza virus (— ● —).

incubated in autologous serum-VBS^{++} did not increase when incubation was continued up to 210 min. Thus, human platelets could activate autologous complement after treatment with influenza virus but not following desialation by bacterial neuraminidase. Therefore, binding and elution of the virus resulted in an additional biochemical alteration of the platelet

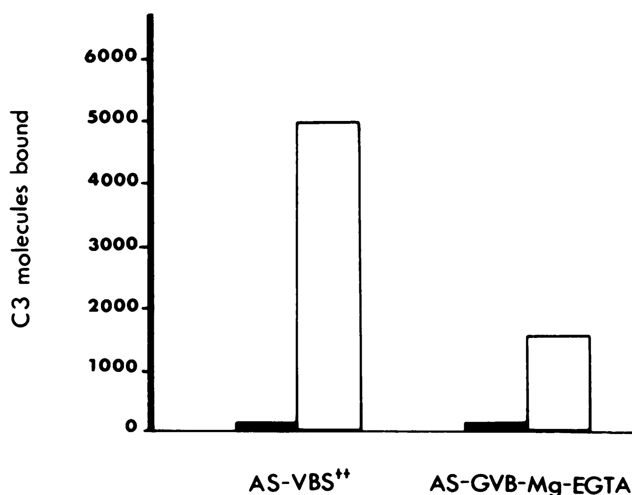

Figure 4. Specific C3 deposition onto *V. cholerae* neuraminidase-treated platelet (■) and virus-treated platelets (□) following incubation for 60 min in autologous serum (AS) diluted in VBS^{++} or in GVB-Mg EGTA. Results are expressed as the number of C3 molecules bound/platelet. Untreated platelets incubated in AS-GVB-MgEGTA bound 3,200 and 2,140 C3 molecules/platelet, respectively. These values were subtracted from the total binding of purified anti-C3 to the treated platelets to calculate the specific deposition depicted in the figure.

membrane than that with desialation, which was responsible for antibody-dependent lysis of pl-v in autologous serum.

Virus-induced modifications of the platelet surface. Platelets incubated with viruses or *V. cholerae* neuraminidase were treated with galactose oxidase and sodium [^{3}H]borohydride. The ^{3}H-labeled glycoproteins were separated and analyzed by SDS-PAGE. Coomassie blue staining revealed identical polypeptide profiles for the virus and *V. cholerae* neuraminidase-treated platelets (not illustrated). Fluorography showed extensive ^{3}H-labeling of the major membrane glycoproteins of both platelet preparations (Fig. 5). Little labeling was observed after control incubations performed in the absence of added virus or bacterial neuraminidase. Thus, it was apparent that incubation of platelets with influenza viruses had resulted in sialic acid removal from each of the major surface glycoproteins. One notable feature of the ^{3}H-labeling patterns was the additional presence of a M_r 72,000 band on analysis of nonreduced samples of virus-treated platelets. Following disulfide reduction, this component migrated with a M_r of 55,000. Such a mobility change did not correspond to that of a known platelet membrane glycoprotein, but resembled that of influenza virus hemagglutinin and the HA1 subunit of this protein (25, 35). Blotting experiments were then performed using rabbit anti-whole virus and anti-viral hemagglutinin antisera. The results confirmed that the M_r 72,000 component expressed antigenic determinants of viral hemagglutinin (Fig. 6). After disulfide reduction, the bulk of the anti-hemagglutinin antibodies were observed to bind to the 55,000 subunit (not illustrated). High molecular weight material (Fig. 6, lanes *a* and *b*) revealed by both anti-whole virus and anti-hemagglutinin antibodies on analysis of nonreduced virus-treated platelet proteins was no longer observed after disulfide reduction, suggesting it represented aggregates of hemagglutinin. No specific antibody binding was observed on analysis of *V. cholerae* neuraminidase-treated platelets and in all cases when using nonimmune rabbit sera. Thus, membranes of virus-treated platelets express antigenic determinants of viral hemagglutinin that remain bound to the platelet membrane after viral treatment of the cells.

Increased lytic activity of a human serum containing an elevated titer of anti-hemagglutinin antibodies. The possibility that lytic antibodies to influenza virus-treated platelets were directed against viral hemagglutinin that was bound to the platelet membrane was then examined by incubating virus-treated platelets in autologous serum that was obtained before and 2 wk after inoculation of a healthy donor with influenza vaccine made of inactivated Bangkok H3N2 and Texas H1N1 A strains and Singapur B strain. After vaccination, serum HAI antibodies to the H3N2 strain rose from a titer of 1:10 to 1:80. Serum total complement hemolytic activity remained unchanged. In parallel, the lytic capacity of the serum towards virus-treated platelets increased so that 55% of the platelets were lyzed and no residual complement hemolytic activity was found in postvaccinal serum in VBS^{++} after 130-min incubation, whereas only 19% of pl-v were lyzed and 31% CH50 was consumed in prevaccinal serum under the same conditions. Absorption of postvaccinal serum with influenza A virus removed all HAI antibodies against H3N2 virus without altering the CH50 and suppressed the lytic activity of the serum for virus-treated platelets.

Discussion

The attachment of myxoviruses to the membrane of erythrocytes, leukocytes, and platelets involves a viral hemagglutinin and specific membrane receptors bearing a terminal *N*-acetyl neuraminic acid group (11, 36). As first shown by Terada et al. (1), saturating amounts of influenza A virus bind to washed human platelets at 4°C and are eluted upon incubation at 37°C through cleavage of membrane receptors by viral neuraminidase. Virus treatment of the platelets did not alter the

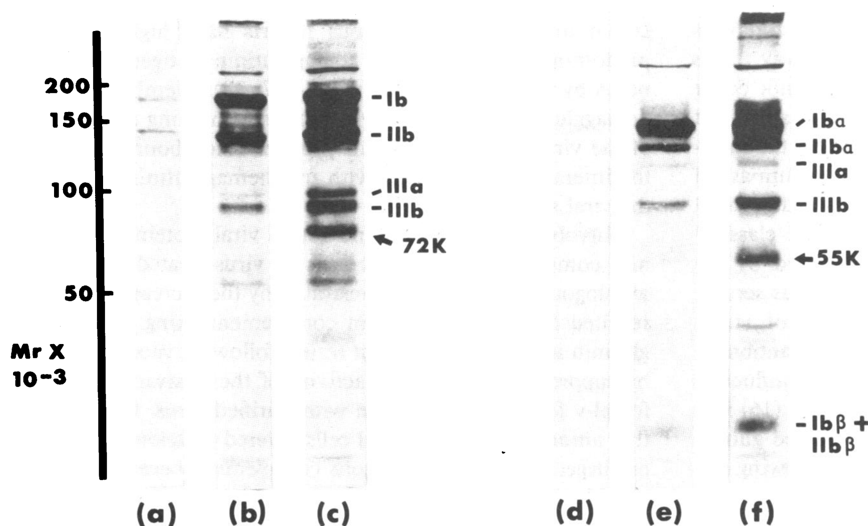

Figure 5. Effect of influenza virus on platelet surface glycoproteins. Washed platelet suspensions were incubated without addition (*a, d*), in the presence of 1 IU *V. cholerae* neuraminidase (*b, e*), or following addition of 20,000 HAU of purified virus (*c, f*), as detailed in Methods. A ^{3}H-label was then introduced into desialated glycoproteins by sequential treatment of the platelets with galactose oxidase and sodium [^{3}H]borohydride. Following solubilization with SDS, samples were analyzed by electrophoresis on 7–12% gradient acrylamide slab gels. Samples were electrophoresed in the absence (*a–c*) and following disulfide reduction (*d–f*). The ^{3}H-labeled glycoproteins were detected in dried, Coomassie blue stained gels by fluorography.

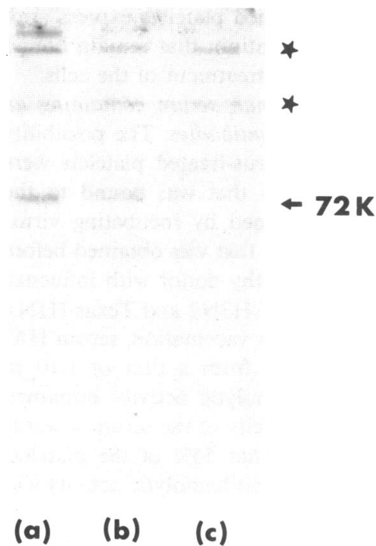

Figure 6. Detection of viral hemagglutinin among platelet polypeptides by immunoblotting. Washed platelet suspensions were incubated with purified influenza virus as in Fig. 5. No ^{3}H-labeling was performed. SDS-solubilized samples were electrophoresed in the absence of disulfide reduction on nitrocellulose membrane and individual strips incubated with (*a*) antiserum against purified viral hemagglutinin, (*b*) anti-whole virus antiserum, and (*c*) nonimmune serum. Bound immunoglobulin was detected by ^{125}I-protein A and autoradiography. *Nonidentified bands given by nonimmune serum.

disc-shape appearance of the cells and did not result in serotonin release or cell lysis. However, incubation of the virus-treated platelets in autologous serum resulted in platelet lysis whereas untreated platelets did not lyze. The amount of lysis, which slightly differed from one donor to another, reached ~10% after 1 h of incubation and 40–50% after 3 h. C3 deposition onto and lysis of virus-treated platelets were predominantly dependent on activation of the classical complement pathway although limited C3 deposition and lysis occurred through activation of the alternative pathway. Lysis also required the presence of antibody since it did not occur in allogeneic agammaglobulinemic serum unless it was restored with immunoglobulins. Lysis was not dependent on the interaction of natural antibodies with neoantigenic sites unmasked by virus-induced desialation of the cells, as platelets desialated with *V. cholerae* neuraminidase did not activate the classical or the alternative complement pathways as assessed by C3 deposition on the cells and did not lyze in autologous serum. Thus, complement- and antibody-dependent lysis of virus-treated platelets in autologous serum differs from both antibody-independent alternative pathway-dependent lysis of influenza virus-treated guinea pig erythrocytes in human serum (16) and from classical pathway-dependent lysis of virus-treated guinea pig erythrocytes in guinea pig serum that occurs following the interaction of natural antibodies with neoantigens expressed by desialated cells (17, 18).

Enzymatic desialation of human platelets using *V. cholerae* neuraminidase resulted in a maximal 55% release of the total platelet-associated sialic acid, whereas the maximal desialation achieved with influenza virus neuraminidase was 45%, indicating that most linkages between terminal *N*-acetyl neuraminic acid and carbohydrates on the human platelet membrane are 2–3 and/or 2–8 linkages whereas about half of the linkages are 2–6 in erythrocytes (16).

Since lysis of virus-treated platelets in autologous serum was not a consequence of virus-induced desialation of the cells, SDS-PAGE analysis of membrane glycoproteins from treated platelets was performed to assess additional alterations in the platelet membrane that could have resulted from adsorption/elution of the virus to and from the cells. Virus-treated platelets were incubated with galactose oxidase and sodium [^{3}H]borohydride so that a ^{3}H-label was introduced into surface galactose and *N*-acetylgalactosamine residues exposed after sialic acid removal. SDS-PAGE under nonreducing conditions was followed by fluorography which revealed the presence of a M_r 72,000 glycoprotein absent from untreated platelets and those incubated with *V. cholerae* neuraminidase. Upon reduction, this component migrated with an apparent M_r of 55,000. Blotting experiments using specific antisera confirmed that these bands represented viral hemagglutinin and the HA1 subunit of this protein. Viral hemagglutinin consists of two disulfide-linked subunits, HA1 ($M_r \simeq 50{,}000$) and HA2 ($M_r \simeq 25{,}000$) (35). Consistent with our findings is the localization of the bulk of the bound carbohydrate on the HA1 chain and the large proportion of galactose and *N*-acetylgalactosamine residues within its oligosaccharides (37). The fact that the hemagglutinin was ^{3}H-labeled confirmed its localization on the surface of the virus-treated platelets. Since both anti-whole virus and anti-hemagglutinin antibodies revealed a single major component, our studies suggest that hemagglutinin is the dominant antigen remaining on the treated platelets. The decreased recognition by anti-hemagglutinin antibodies of the M_r 25,000 HA2 subunit of hemagglutinin is not unexpected as previous reports have highlighted the predominant expression of hemagglutinin antigenic determinants by the HA1 subunit of HA (37, 38). Membrane-bound hemagglutinin could represent protein remaining after elution of the virus from the cells, or protein which bound following the interaction of platelets with free hemagglutinin present in the viral suspension.

Involvement of membrane-bound viral protein in antibody and complement-dependent lysis of virus-treated platelets in autologous serum was demonstrated by the increased lysis that resulted from the increase in complement-fixing anti-hemagglutinin antibodies in the test serum following vaccination and by suppression of the lytic activity of the postvaccinal serum for pl-v following adsorption with purified virus. It is possible that among peripheral blood cells, altered platelets represent a privileged target for autologous complement because they are capable of uptake of the terminal complement C5b-9 complex (39) and because they lack the C3b receptor that is a potent

inhibitor of complement activation. Accelerated clearance of platelets following their contact with influenza virus (1, 3), and possibly, clearance of other cells on which influenza A antigen can be demonstrated in vivo during peak infection with influenza (40) could result from both antibody and complement-mediated lysis, and from antibody-dependent C3 deposition that would result in the interaction of the target cell with C3b receptors on cells from the reticuloendothelial system (41). The prevalence of anti-hemagglutinin antibodies in the normal population is very high. Following infection with influenza virus, interstrain cross-reactive complement fixing anti-hemagglutinin antibodies are the major feature of the ongoing immune response. Binding of a viral protein to integral membrane glycoproteins after contact of human platelets with influenza virus or with viral hemagglutinin provides a model for immune thrombocytopenias occurring during acute viral infections at the time of the specific immune response.

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