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Characterization of immunoglobulin binding to isolated human erythrocyte membranes: evidence for selective, temperature-induced binding of naturally occurring autoantibodies to the cytoskeleton

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Abstract

Human plasma contains naturally occurring autoantibodies to the predominant components of the erythrocyte membrane: band 3 and spectrin bands 1 and 2 of the cytoskeleton. The titer of cytoskeletal plasma autoantibodies increases in various hemolytic conditions, suggesting that opsonization of the cytoskeleton may play an important role in the clearance of hemolyzed (not senescent) erythrocytes from the circulation. In this study, we use Alexa Fluor 488 goat anti-human IgG conjugate (Molecular Probes, Eugene, OR, USA), to characterize plasma immunoglobulin binding to erythrocyte membranes from osmotically hemolyzed cells ('ghosts'). The results show that exposure of ghosts to plasma results in 4-fold more immunoglobulin binding to the cytoskeleton than is bound to the proteins contained within the lipid bilayer. Preincubation of the ghosts at 37°C causes 8-fold more immunoglobulin binding to the cytoskeleton compared to bilayer proteins. This temperature-induced change resulted from selective immunoglobulin binding to the cytoskeleton, with no change in immunoglobulin binding to bilayer proteins. However, the rate of increase in cytoskeletal antigenicity at 37°C did correlate with the rate of a conformational change in band 3, a transmembrane protein which serves as a major membrane attachment site for the cytoskeleton. The results of this study suggest that the cytoskeleton is the primary target in the opsonization of hemolyzed erythrocyte membranes by naturally occurring plasma autoantibodies. The conformational changes which occur in ghosts at 37°C are associated with selective exposure of new immunoglobulin binding sites on the cytoskeleton, and with a change in the structure of band 3. We propose a model suggesting that opsonization of the cytoskeleton occurs prior to the decomposition of hemolyzed erythrocytes at 37°C.

Keywords: Immunoglobulin; Fluorescence; Band 3; Cytoskeleton; Hemolysis; Erythrocyte; Homeostasis

1. Introduction

Human plasma contains specific IgG autoantibodies against the major components of the erythrocyte membrane: band 3 (the erythrocyte anion exchanger) [1,2], and spectrin bands 1 and 2 of the cytoskeleton [3]. Since intravascular hemolysis results in the re-

Abbreviations: $C_{12}E_8$, poly(oxyethylene-8-lauryl ether); $C_{12}E_9$, poly(oxyethylene-9-lauryl ether); DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; IGAF488, anti-human goat IgG conjugated to Alexa Fluor 488; TLCK, *Nα-p*-tosyl-L-lysine chloromethyl ketone; PMFS, phenylmethylsulfonyl fluoride

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lease of hemoglobin and other cellular components into the plasma, and is expected to allow plasma proteins to enter the hemolyzed cell, it is probable that the cytoskeletal autoimmunity plays a significant role in removal of such hemolyzed cells from the circulation. Yet, most experimental work in the literature has focused on the mechanism of band 3 antigenicity in senescent, intact erythrocytes [4–9], where cytoskeletal antigenicity would not be an issue. In the study by Lutz and Wipf [3], an immunoelectrophoretic method using SDS-solubilized membranes, and immunoprecipitation of isolated spectrin were used to characterize binding of naturally occurring autoantibodies. There is no information available on the mechanisms associated with the development of cytoskeletal antigenicity within hemolyzed, but otherwise intact cells. For example, it is not known whether plasma autoantibodies can even bind to the cytoskeleton when it is associated with the lipid bilayer.

In this report, we present the first experiments which characterize plasma immunoglobulin binding to the major membrane components of osmotically hemolyzed erythrocytes. We have studied the effect of temperature on plasma immunoglobulin opsonization of unsealed human erythrocyte membrane ghosts using anti-human goat IgG labeled with Alexa Fluor 488 (IGAF488) (Molecular Probes, Eugene OR, USA), which reacts with human IgG heavy chains, and with all classes of human immunoglobulin light chains. The results show that the cytoskeleton is the predominant immunoglobulin binding site on the erythrocyte membrane in hemolyzed cells, and that the amount of immunoglobulin binding to the cytoskeleton increases when ghosts are preincubated at 37°C. This increase correlates with the conformational change in band 3 from a transport active to an inactive form. These results are discussed within the context of a new model for opsonization and decomposition of hemolyzed erythrocytes at 37°C.

2. Materials and methods

2.1. Preparation of unsealed ghosts

Unsealed ghosts were prepared as described previously [10] with the following specific modifications. Fresh venous blood was drawn in heparinized tubes and centrifuged. The plasma was collected and saved for later use (see below). Forty-five day old packed erythrocytes were obtained from the Omaha Chapter of the American Red Cross. O^+ plasma was obtained from the Blood Bank of the University of Nebraska Medical Center. Erythrocytes were washed four times in PBS (5 mM sodium phosphate/150 mM NaCl (pH 8)) at 4°C, and the buffy coat was removed by aspiration. Stoichiometric labeling of band 3 in intact erythrocytes by DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) was performed as described previously [10]. Samples were incubated for 45 min at 37°C and 50% hematocrit in PBS (hematocrit is defined in this paper as the volume of packed cells or ghosts per total sample volume×100), or in 25 μM DIDS in PBS. Cells were washed once in PBS with 0.5% bovine serum albumin at 4°C, and then four more times in PBS at 4°C. Unsealed ghosts were prepared from these DIDS-labeled, or from unlabeled cells by osmotically hemolyzing them with a 1/18 dilution in ice cold 5P8 (5 mM sodium phosphate (pH 8)), washing the ghosts three times in 5P8, and then once in 20P8 (20 mM sodium phosphate (pH 8)), all in a refrigerated centrifuge at 4°C.

2.2. Preparation of resealed ghosts

Resealed ghosts were prepared essentially as described previously [10], with the following specific modifications. Six milliliters of washed erythrocytes were osmotically hemolyzed 1:5 in ice cold 5P8. The sample was centrifuged at 4°C, and the hemolysate collected. Salts and buffer were added to this cold hemolysate, and this mixture was added back to the unsealed ghosts to give the following final concentrations: 90 mM sodium sulfate, 5 mM sodium phosphate, 5 mM Bis-Tris (*N*,*N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane), 5 mM Tris (tris(hydroxymethyl)aminomethane), 1 mM magnesium sulfate, with a final pH of 7.4. Resealing was initiated by incubating at 37°C for 1 h. The resealed ghosts were then washed in the Bis-Tris/Tris/sulfate buffer three times.

2.3. Incubation studies with unsealed or resealed ghosts

2.3.1. Unsealed ghosts

Fifteen milliliter conical tubes were coated with the protease inhibitors PMSF (phenylmethylsulfonyl fluoride), pepstatin A and leupeptin, by adding methanol solutions containing these inhibitors, and evaporating the methanol as described previously [10]. White unsealed ghosts were placed into these pretreated tubes and brought to 50% hematocrit with 20P8. The tubes were sealed and placed in the refrigerator overnight to inhibit resealing in subsequent experimental steps [11,12]. The next day, part of this sample was taken for analysis and designated as the zero time point. The remainder of the sample was placed in a tube pretreated with protease inhibitors, and then placed in a water bath at 37°C.

It is known that incubation of ghosts at 37°C in low ionic strength buffer leads to their breakdown to vesicles [12,13]. Such breakdown can be minimized by suspending ghosts in higher ionic strength buffer [13]. We chose to incubate the ghosts in 20P8, which was high enough in ionic strength to minimize the breakdown of the ghosts to vesicles, as evidenced by the fact that the unsealed ghost suspensions could be completely pelleted by centrifugation at $31\,000 \times g$ for 15 min, to yield typical 'fluffy' white ghosts. In our experience, a longer centrifugation time (at least 30 min) is required to bring down vesicles using the same g force.

2.3.2. Resealed ghosts

Resealed ghosts were prepared as described above, and were placed in a 15 ml conical tube pretreated with inhibitors as just described. The sample was stored overnight at 4°C. The next day, a zero time point was taken for analysis, with the remainder placed in the 37°C water bath.

2.4. Measurement of the extent of sealing during incubation of pretreated unsealed ghosts at 37°C

The ability of trypsin to selectively cleave the cytoplasmic domain of band 3 [14] was used to measure the extent of resealing of unsealed ghosts which had been preincubated overnight at 4°C, and then incubated at 37°C. The assumption in this experiment is that trypsin cleavage of the cytoplasmic domain will only occur in unsealed ghosts, and that inhibition of such cleavage is indicative of ghost resealing. Unsealed ghosts were prepared as described above, and incubated overnight at 4°C. They were then incubated for various lengths of time at 37°C. Aliquots were removed, washed in 5P8 at 4°C to remove the protease inhibitors, and then treated with trypsin as described in previous reports [14,15]. The trypsin reaction was stopped by addition of 25 μg/ml of TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone), plus 1 mM PMSF. The samples were then washed at 4°C, and solubilized in SDS for SDS-PAGE, all as described previously [15].

2.5. Treatment of ghosts with plasma, and labeling with IGAF488

2.5.1. Unsealed ghosts

Two hundred microliters of unsealed ghosts (50% hematocrit) were taken either from the samples incubated at 4°C, or from samples incubated at 37°C for various times, and placed in 50 ml centrifuge tubes. Fifteen milliliters of buffer A (10 mM HEPES, 130 mM NaCl, pH 7.4) were added to each tube, and they were centrifuged at 4°C, for 15 min at $31\,000 \times g$. This wash was repeated, after which 3.3 ml of plasma was added to each 100 μl of ghosts (final hematocrit = 3%). These samples were incubated for 1 h at 37°C. The tubes were then filled to 35 ml with buffer A and centrifuged for 1 h at $31\,000 \times g$ and 4°C. They were then aspirated and washed two more times in buffer A at 4°C. IGAF488 was always centrifuged for 4 min at 15 000 rpm in a TOMY MC-150 High Speed Microcentrifuge to minimize non-specific binding (Molecular Probes) (also see below). Three hundred microliters of 0.1 μM IGAF488 in buffer A were then added to each 100 μl of ghosts. Such samples were then incubated for 2 h at 37°C, after which the tubes were filled to 35 ml with buffer A and centrifuged at 4°C. Samples were washed two more times in buffer A at 4°C. To determine the extent of background binding of IGAF488, we treated ghosts with IGAF488 exactly as just described, except that the plasma treatment step was omitted.

2.5.2. Resealed ghosts

A 250 μl sample of resealed ghosts (50% hematocrit), incubated as described above, was taken at each time point, placed in a 50 ml centrifuge tube, and washed twice in buffer A at 4°C, which maintains ghost sealing status. After the last centrifugation, 3.3 ml of centrifuged plasma was added to 125 μl of these resealed ghosts. The resealed ghosts were incubated for 1 h at 37°C. The 50 ml tube was then filled with buffer A, and centrifuged for 1 h at 31 000×*g* and 4°C. They were washed two more times in buffer A with 15 min centrifugation at 31 000×*g* and 4°C. Three hundred microliters of 0.1 μM centrifuged IGAF488 in buffer A were added to each 125 μl of resealed ghosts. The sample was incubated for 2 h at 37°C, after which the tubes were filled to 35 ml with buffer A and centrifuged at 4°C. The sample was washed two more times in buffer A. Unsealed ghosts were then prepared as described above.

2.6. Isolation of erythrocyte cytoskeletons

The method used for isolation of cytoskeletons has been described extensively in the literature [16–20]. It involves solubilization of ghosts in the non-ionic detergent $C_{12}E_8$ (poly(oxyethylene-8-lauryl ether), followed by centrifugation and washing of the pellet, which contains the cytoskeletons. These washed cytoskeletons were then solubilized in SDS in order to measure total protein (see below), the fluorescence of IGAF488, and the fluorescence of DIDS-band 3 covalent adduct. Specifically, 1.4 ml of ghosts were brought to 4.2 ml (33% hematocrit) in 20P8 plus 1% $C_{12}E_8$. Then 0.3 ml of these solubilized ghosts were withdrawn and scanned in the fluorimeter for IGAF488 and DIDS fluorescence as described above. The remainder of the preparation was incubated on ice for 20 min, and then centrifuged for 30 min at 31 000×*g* and 4°C, to pellet the cytoskeletons. The pellet was gently resuspended in 20 ml of 5P8, and then centrifuged for 30 min at 31 000×*g* and 4°C. The supernatant was removed and scanned to establish the removal of unbound IGAF488. These washed pellets were then solubilized in 1% SDS in 5P8 (3.9 ml final total volume), and scanned for IGAF488 and DIDS-band 3 adduct fluorescence. Protein absorbance was measured at 280 nm.

2.7. Analytical methods

2.7.1. Fluorescence measurements

Unsealed ghosts (100 μl) treated with plasma and IGAF488 were collected and solubilized in a final volume of 300 μl with buffer containing 20P8 and 1% $C_{12}E_8$ (final concentrations). These samples were placed in a fluorescence cuvette with a 10 mm excitation pathlength and a 4 mm emission pathlength. The fluorescence emission spectrum was measured at 25°C using a Perkin-Elmer Model 650-40 fluorescence spectrometer. IGAF488 fluorescence spectra were collected between 460 and 550 nm, with the excitation wavelength held at 495 nm. The fluorescence signal intensity was found to be related linearly to fluorophore concentration under all conditions. DIDS-band 3 fluorescence spectra were collected between 330 and 500 nm, with the excitation wavelength set at 350 nm. DIDS fluorescence does not interfere with IGAF488 fluorescence measurement. Fluorescence data were normalized by measuring the protein absorbance of solubilized samples at 280 nm. An empirical extinction coefficient for solubilized ghosts was determined at this wavelength, using a stock solution of solubilized ghosts and the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), with a standard curve derived from various concentrations of bovine serum albumin. The 280 nm extinction coefficient for solubilized ghosts was 1.07 ml/mg. Thus, a sample of solubilized ghosts containing 0.93 mg/ml protein will give an absorbance of 1.0 at 280 nm.

2.7.2. Gel filtration chromatography

Gel filtration chromatography was performed essentially as described in previous reports from this laboratory [10,15] using Sepharose CL-4B as the matrix. The column buffer contained 100 mM NaCl, 5 mM sodium phosphate, 0.1% $C_{12}E_9$ (poly(oxyethylene-9-lauryl ether)) pH 8. Ghosts treated as described above were washed and pelleted and suspended to final concentrations of 1% $C_{12}E_9$ and about 2–3 mg/ml of ghost protein. Five hundred microliters of this sample were then applied to the column. The absorbance of the column effluent was monitored continuously at 280 nm, using a Pharmacia LKB UV monitor. The first 17 ml of effluent were collected to establish the effluent flow rate, and there-

after, 1 ml fractions were collected. Each fraction was then read for IGAF488 and DIDS-band 3 adduct fluorescence (where indicated). Sample absorbance was measured on each tube directly at 280 nm using an Hitachi Model 100-60 spectrophotometer.

2.7.3. Anion transport measurements

Dithionite-sulfate exchange was measured using the spectrophotometric assay developed in this laboratory as described in a previous report [10]. Velocities were determined at several dithionite concentrations over a range where the transport data followed hyperbolic kinetics. The V_{max} value was determined, and the percent change in V_{max} plotted versus ghost preincubation time at 37°C.

3. Results

3.1. Plasma immunoglobulin binds to unsealed ghosts to a greater extent than to resealed ghosts, and incubation at 37°C increases binding to unsealed but not to resealed ghosts

We studied immunoglobulin binding to unsealed and resealed ghosts. In the unsealed ghost experiments, our approach was to hemolyze erythrocytes osmotically, prepare the ghosts and preincubate them overnight at 4°C, in order to inhibit sealing during subsequent incubation at 37°C [11,12]. Trypsin cleavage studies confirmed that the cytoplasmic domain of band 3 was as accessible to the action of this protease throughout the 120 h incubation process at 37°C as it was in the initial preparation of unsealed ghosts. The gel showed that 100% of the band 3 was cleaved at each time point studied over the 120 h incubation period (data submitted for review).

After incubation, the samples were washed, treated with plasma, and then treated with IGAF488, as described in Section 2. Experiments were performed with either fresh erythrocytes and autologous plasma ($n=7$), or with 45 day old erythrocytes ($n=2$), and non-autologous plasma from an O^+ individual. There was no significant difference in the results obtained with fresh versus 45 day old red cells, or from the use of autologous versus non-autologous plasma.

Fig. 1 shows normalized fluorescence spectra of various ghost samples after solubilization in 20P8 plus 1% $C_{12}E_8$. Unsealed ghosts which were not treated with plasma or IGAF488 gave no fluorescence peak between 550 nm and 505 nm (spectra 1 in Fig. 1A,B). The large increase in the signal below 500 nm in Fig. 1 (curve 1) is due to the breakthrough from the excitation source.

Treatment of unsealed ghosts with plasma followed by IGAF488 yielded a fluorescence spectrum for detergent solubilized ghosts which had a peak centered around 515 nm (Fig. 1A, spectrum 2). Treatment with IGAF488 alone (no plasma treatment step) gave a background binding which was about 3–5-fold smaller than that seen for spectrum 2 in Fig. 1A (not shown, but see the discussion of Fig. 2 below). Incubation of unsealed ghosts at 37°C for 120 h increased the amount of IGAF488 bound per mg of total ghost protein by 2.5-fold (compare spectra 2 and 3 in Fig. 1A). Electrophoresis of the samples showed that the incubation under our conditions in the presence of protease inhibitors caused no significant change in the classical pattern for Coomassie blue stained SDS-PAGE gels of unsealed ghosts [16] (data not shown).

Fig. 1B illustrates that when the same unsealed ghost preparations were incubated for 120 h at 4°C, no increase in immunoglobulin binding was observed (compare spectrum 2 to spectrum 3 in Fig. 1B). Keep in mind that the immunoglobulin binding assay (i.e. the plasma and IGAF488 treatment steps) was always performed under exactly the same conditions for the various experiments shown in Fig. 1A,B (see Section 2). Thus, increased immunoglobulin binding to unsealed ghosts is dependent on the temperature experienced by the ghosts prior to being exposed to plasma and IGAF488.

An important question is whether increased immunoglobulin binding involves sites on the outside, or on the inside of the ghosts. Fig. 1C shows the results from studies with resealed ghosts incubated at 37°C. In this case, the resealed ghosts were incubated for 144 h, and then treated with plasma and IGAF488. Resealed ghosts showed no increase in immunoglobulin binding after 144 h (Fig. 1C, compare spectrum 2 to spectrum 3). In addition, the initial amount of IGAF488 bound per mg of ghost protein was substantially less than that seen when unsealed ghosts were treated with plasma and IGAF488 (compare

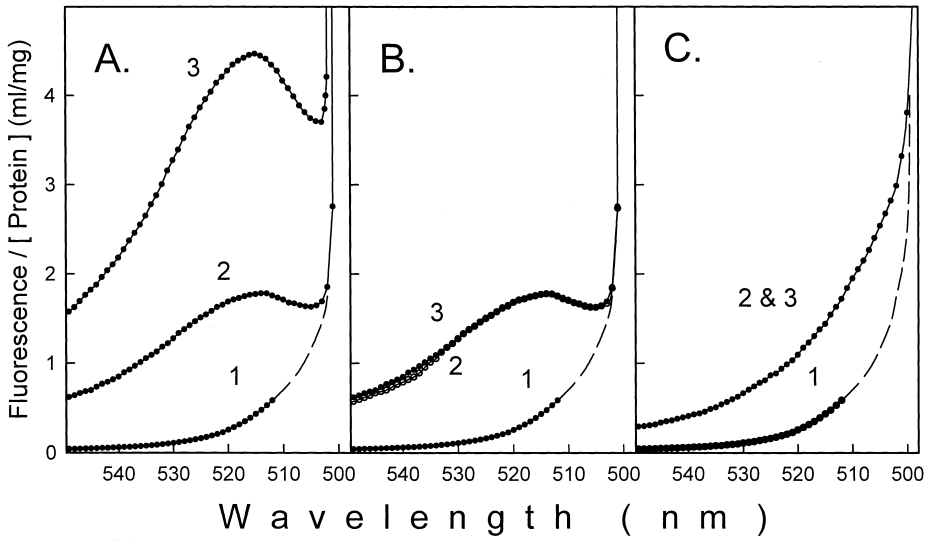

Fig. 1. Fluorescence spectra of solubilized unsealed ghosts incubated at either 4°C, or 37°C, or of resealed ghosts incubated at 37°C, and then opsonized with plasma immunoglobulin and IGAF488. Ghosts were treated as described below, and then solubilized in 20P8 plus 1% $C_{12}E_8$, as described in Section 2. The fluorescence spectra were collected at 25°C ($\lambda_{excite} = 495$ nm) as continuous traces on a strip chart recorder. The data points were read from the traces and each spectrum was normalized by dividing by the total protein concentration for the given sample. Total protein was determined by measuring the absorbance at 280 nm, and using an experimentally determined extinction coefficient for total ghost protein (see Section 2 for further details). (A) Unsealed ghosts incubated at 37°C. Spectra: 1, initial unsealed ghost sample, no plasma or IGAF488 added; 2, initial unsealed ghost sample treated with plasma and IGAF488; 3, unsealed ghosts incubated for 120 h at 37°C, and then treated with plasma and IGAF488. (B) Unsealed ghosts incubated at 4°C. Spectra: 1, initial unsealed ghost sample with no added plasma or IGAF488; 2, initial unsealed ghost sample treated with plasma and IGAF488; 3, unsealed ghosts incubated for 120 h at 4°C, and then treated with plasma and IGAF488. (C) Resealed ghosts incubated at 37°C. Spectra: 1, no added plasma or IGAF488; 2, initial resealed ghosts treated with plasma and IGAF488; 3, resealed ghosts incubated at 37°C for 144 h, and then treated with plasma and IGAF488. The large peak ($\lambda < 500$ nm) in all of the panels is the breakthrough from the excitation source at 495 nm.

the spectra labeled 2 in Fig. 1A–C). However, the fluorescence intensity around the IGAF488 peak was greater for resealed ghosts treated with plasma and IGAF488 than for ghosts which had not been exposed to plasma or IGAF488. Thus, plasma immunoglobulin binding sites do exist on the outer surface of ghosts (also see below). Substantially more sites exist on the inner surface of the ghosts (under the same plasma treatment conditions), and the increase in immunoglobulin binding to unsealed ghosts upon incubation at 37°C involves sites at the inner surface of the membrane.

3.2. The erythrocyte cytoskeleton is the predominant plasma immunoglobulin binding site on the cytoplasmic surface of unsealed ghosts

The cytosolic side of unsealed ghosts contains two potential immunoglobulin binding sites which exist in sufficient quantity to explain our observations. One of these sites is the cytoplasmic domain of band 3, while the other is the cytoskeleton. We used classical means to separate the cytoskeleton from the major components contained within the lipid bilayer, i.e. solubilization of the ghosts in nonionic detergent at relatively high ionic strength (20P8), followed by centrifugation, removal of the supernatant and washing of the pellet [17–20]. The ghosts were derived from erythrocytes which were labeled with DIDS (a covalent, band 3-specific anion transport inhibitor), so that we could follow the disposition of band 3 in subsequent gel filtration studies.

Background IGAF488 binding to unsealed ghosts not exposed to plasma (Fig. 2, M Initial (−) plasma) was 5-fold lower than IGAF488 binding to ghosts which were pretreated with plasma (Fig. 2, M Initial (+) plasma). Such background binding of IGAF488 to untreated ghosts was found to be located exclusively on the components contained within the

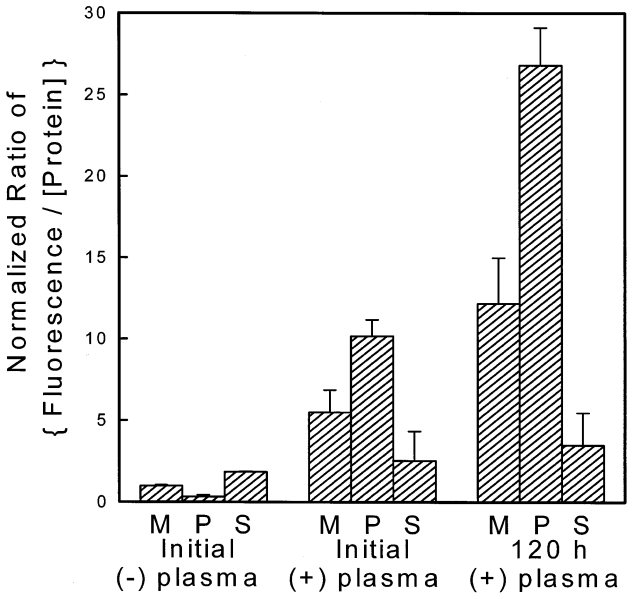

Fig. 2. Normalized ratios of bound IGAF488 (fluorescence/protein) for solubilized DIDS-labeled unsealed ghosts (M), for the washed, solubilized cytoskeletal pellet (P), and for the supernatant fraction (S). Unsealed ghosts from initial samples or from samples incubated for 120 h at 37°C were either treated or not treated with plasma, and then all samples were treated with IGAF488 as described in Section 2. These samples were solubilized in 20P8 plus 1% $C_{12}E_8$. Part of the sample was saved as the total membrane sample for each experimental condition (M). The remainder of each sample was centrifuged and the supernatant fraction (S) was removed. The pellet (P) was washed once in 5P8 and saved. All three types of samples (M, P, S) were analyzed by SDS-PAGE, by measuring the fluorescence of IGAF488, and by measuring the protein absorbance at 280 nm, as described in Section 2. The normalized ratios of IGAF488 fluorescence/[protein] were calculated by first determining the absolute fluorescence/[protein] ratio for a given sample as in Fig. 1, and then dividing that ratio by the ratio for M Initial (−) plasma. IGAF488 binding is observed for the initial sample and is confined entirely to the supernatant fraction, with no detectable fluorescence in the cytoskeletal fraction compared to background (no additions). Pretreatment of ghosts with plasma caused a 5-fold increase in IGAF488 binding to the ghosts (M initial (−) versus (+) plasma) which is due to increased binding to the cytoskeleton. Incubation of ghosts for 120 h at 37°C caused a slight increase in IGAF488 binding in the absence of plasma (see Fig. 4B), but caused a 2-fold increase in IGAF488 binding to ghosts (M initial (+) plasma versus M 120 h (+) plasma). This increased binding with temperature and time occurs predominantly due to immunoglobulin (IGAF488) binding to the cytoskeleton.

detergent-soluble membrane fraction of ghosts (Fig. 2, S Initial (−) plasma). The pellet fraction from ghosts treated with IGAF488 alone (Fig. 2, P Initial (−) plasma) had no detectable IGAF488 bound compared to untreated controls (also see Fig. 4B below). While pretreatment with plasma caused a slight increase in IGAF488 binding within the solubilized membrane fraction (Fig. 2, S Initial (+) plasma versus S Initial (−) plasma), there was a 20-fold increase in the amount of bound IGAF488 within the pellet fraction (Fig. 2, P Initial (−) plasma versus P Initial (+) plasma).

Incubation of ghosts at 37°C for 120 h, followed by treatment with plasma and IGAF488, caused an approx. 2-fold increase in IGAF488 binding to membranes as compared to initial unsealed ghosts (Fig. 2, M Initial (+) plasma versus M 120 h (+) plasma). This result was not significantly different from that seen for unsealed DIDS-free ghosts (Fig. 1A, spectrum 2 versus spectrum 3). The increased immunoglobulin binding after incubating for 120 h at 37°C is almost exclusively due to immunoglobulin binding to the cytoskeletal pellet (Fig. 2, compare 120 h (+) plasma, P versus S). It should be noted that the cytoskeletons so generated were free of band 3 on the basis of DIDS fluorescence measurements, and that they contained spectrin and actin as predominant Coomassie blue staining bands on SDS-PAGE (data not shown).

We next analyzed band 3 in the solubilized ghosts by analytical gel filtration chromatography [8,10,15] to see if immunoglobulin binding to band 3 changes during incubation, and to determine if the aggregation state of band 3 changes. Fig. 3 shows chromatographic profiles for a sample of membranes after incubation for 120 h at 37°C, and for a sample of the same membranes without incubation. The elution profiles using DIDS fluorescence (top panels in Fig. 3) indicate the position of band 3. There was no DIDS fluorescence in the void volume for either the initial or the 120 h incubated samples, indicating that band 3 maintained its dimeric/tetrameric state over this time period, and did not aggregate to higher oligomers, or dissociate to monomers, in agreement with our previous studies [10].

The lower panels in Fig. 3 show the corresponding profiles for protein concentration (closed circles), and for IGAF488 (Alexa) fluorescence (open circles). Gel

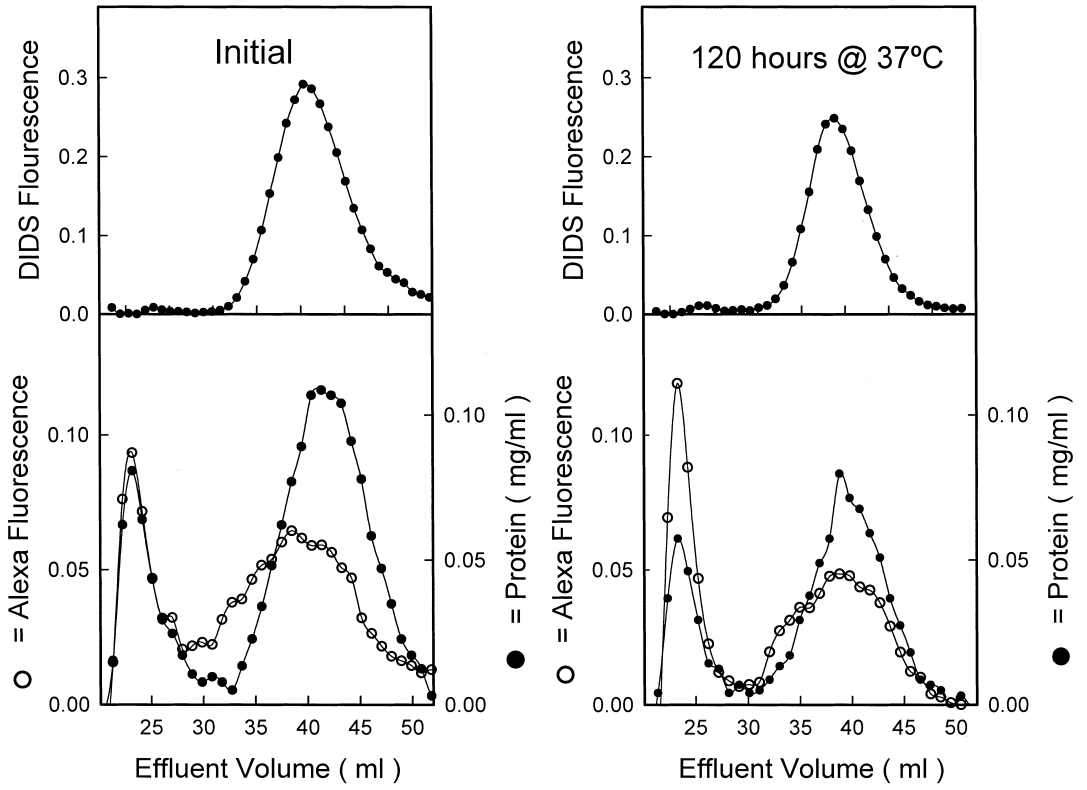

Fig. 3. Gel filtration chromatography of initial, and 120 h, 37°C incubated unsealed ghost samples, treated with plasma and IGAF488, and then solubilized in non-ionic detergent. Ghosts from DIDS-labeled erythrocytes were prepared, and an initial sample taken. The remainder of the material was incubated for 120 h at 37°C as described in Section 2. Both sets of ghosts were treated with plasma and IGAF488, solubilized in $C_{12}E_8$, and these solubilized samples applied to a CL-4B column as described in Section 2. Tubes were collected and analyzed for protein absorbance at 280 nm, IGAF488 fluorescence, and DIDS fluorescence.

electrophoresis (not shown) indicated the presence of certain cytoskeletal proteins in the void volume, but no band 3, in agreement with the DIDS fluorescence profile. Band 3 (Coomassie blue staining) and glycophorin A (periodic acid Schiff's base staining) were observed in the main peak of the included volume. Glycophorin A was present in the trailing edge of the peak, while band 3 was present across the peak, but most prominently in the leading edge and at the peak position.

In order to quantitate changes in immunoglobulin binding to band 3, we measured the integrated peak area of the DIDS fluorescence profile and the peak area of the IGAF488 fluorescence profile. The ratio of IGAF488 to DIDS fluorescence was 0.27 for the initial sample in Fig. 3. It was 0.25 for the same preparation of membranes after incubation for 120 h at 37°C. While the ratios for band 3 were not significantly different, there was an increase in the ratio of IGAF488 fluorescence to protein for the material in the void volume, which contained cytoskeletal proteins, but no band 3.

3.3. *Correlation of the kinetics of band 3 conformational changes with the kinetics of increased immunoglobulin binding to the cytoskeleton*

While incubation of ghosts at 37°C does not promote increased binding of plasma immunoglobulins to band 3, we had shown previously that the membrane domain of band 3 does undergo a conformational change over this time range when incubated at 37°C [10]. Since band 3 is a major attachment site for the cytoskeleton, we investigated whether there was a correlation at 37°C between the rate of increase in

immunoglobulin binding to the cytoskeleton and the rate of change in band 3 from the transport-active to the less active form [10]. The incubation conditions used here were not exactly the same as in our previous study (i.e. there was no dithiothreitol used here), so we elected to measure the rate of band 3 conformational change under the present conditions, using a transport assay [10], and to correlate this with kinetic measurements of the increase in immunoglobulin binding to unsealed ghosts incubated for various times at 37°C. Resealed ghosts were used to measure anion transport. In separate experiments, the spectral shift in DIDS-band 3 fluorescence [10] was also measured under our present conditions.

The kinetics of the increase in immunoglobulin binding to unsealed ghosts incubated at 37°C were studied at various times. Fig. 4A shows that the immunoglobulin binding capacity increases by about 2.5-fold, to an apparent end point, following an exponential time course with an apparent rate constant of 0.012 h^{-1} (see legend to Fig. 4). Fig. 4B indicates

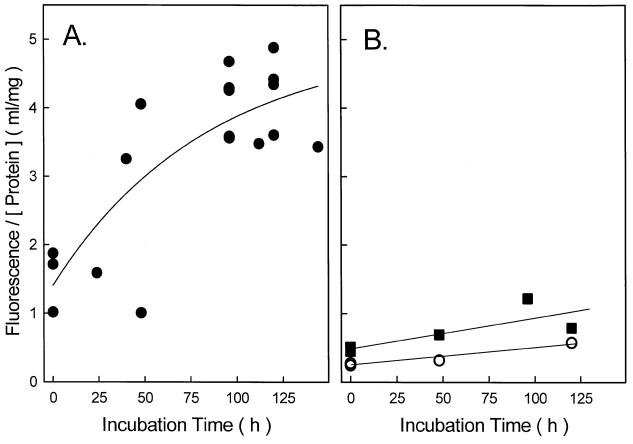

Fig. 4. Kinetics of increase in plasma immunoglobulin binding to unsealed ghosts incubated at 37°C. Unsealed ghosts were incubated at 37°C, and samples taken at various times. Samples were split into three portions and treated: (A) with plasma and IGAF488 (●); (B) with no additions (○), or with IGAF488 alone (■). IGAF488 fluorescence and protein absorbance were determined on the solubilized treated samples as described in Fig. 1. The points are independent determinations. The data in A were fit to a single exponential function of the form: $F = F_\infty(1-e^{-kt}) + F_0(e^{-kt})$, where F is the ratio of fluorescence, at 515 nm, to protein concentration, and k is the apparent rate constant. We obtained values of $F_0 = 1.24 \pm 0.63$ ml/mg, $F_\infty = 4.6 \pm 2.01$ ml/mg, and $k = 0.012 \pm 0.01$ h^{-1}. The curve in A indicates the fit. The lines in B are fits to a linear function.

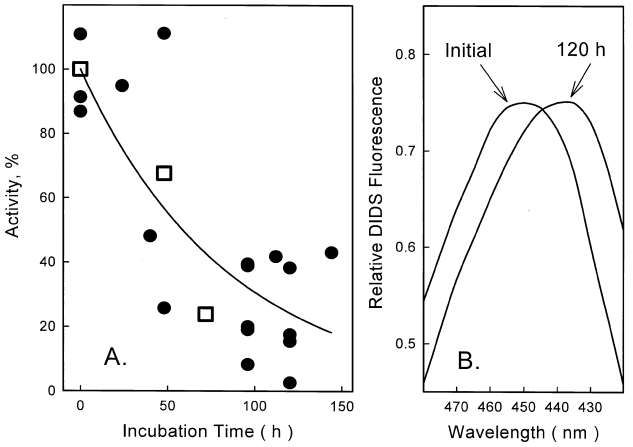

Fig. 5. Correlation of band 3 conformational changes with plasma immunoglobulin binding. (A) Plot of the loss in transport activity of resealed ghosts ($100\times(V_{max}/(V_{max})_{initial})$) (□) as one measure of band 3 conformation [10], and of the percent remaining immunoglobulin binding sites present in unsealed ghosts (●). Percent remaining immunoglobulin binding sites was calculated from the data in Fig. 4A as $100\times(F-F_\infty/F_0-F_\infty)$, where F is defined as in Fig. 4. The curve through the data is drawn on the basis of an exponential fit to the immunoglobulin binding data of Fig. 4A. (B) Fluorescence spectra of DIDS-labeled membranes derived from initial ghosts, or from ghosts incubated for 120 h at 37°C, and solubilized in $C_{12}E_8$.

that incubation of ghosts at 37°C promotes only about a 30% increase in binding of IGAF488 to unsealed ghosts incubated for various times at 37°C, when the plasma treatment step is omitted. We saw in Fig. 2 that plasma-independent IGAF488 binding occurs exclusively to components in the membrane domain.

Fig. 5A shows a plot of the percent of initial transport V_{max} remaining (i.e. $100\times(V_{max}/(V_{max})_{initial})$) for resealed ghosts as a function of incubation time (open squares) versus the percent of remaining immunoglobulin binding sites (closed circles). The data for immunoglobulin binding were taken from Fig. 4A. The number of immunoglobulin binding sites increases with time (Fig. 4A). In Fig. 5A we plot the percent of immunoglobulin binding sites remaining in a given sample, which was calculated using the estimated value for the maximum in immunoglobulin binding from the fit in Fig. 4A as the 100% value (see legend to Fig. 5). The loss of transport activity occurs at a rate which is similar to the rate of appearance of new immunoglobulin binding sites on the

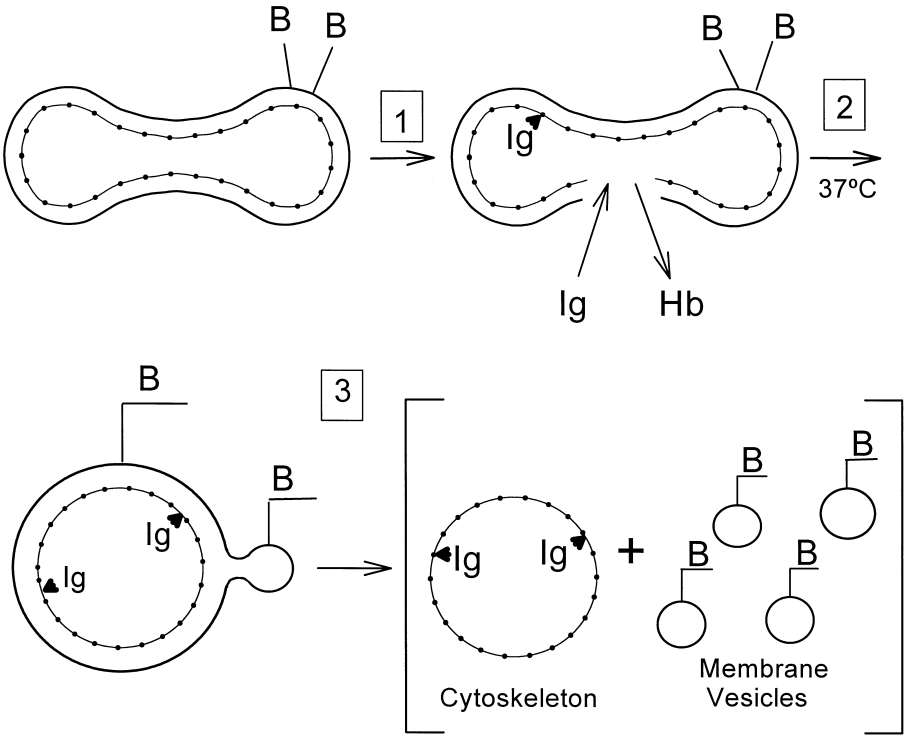

Fig. 6. Schematic model for the in vivo opsonization and decomposition of the hemolyzed human erythrocyte at 37°C. This schematic shows: (1) the hemolysis of a single erythrocyte, and the release of hemoglobin (Hb) and the entrance of plasma immunoglobulin (Ig); (2) the conformational change in band 3 (B) from native (perpendicular to the surface of the membrane) to the transport inactive form (bent conformation), and the increase in binding of immunoglobulin to the cytoskeleton, combined with migration of band 3 into budding bilayer vesicles; which leads to (3) the final product, consisting of a 'stripped', immunoglobulin saturated cytoskeleton, and cytoskeleton-free membrane vesicles. See text for further details.

cytoskeleton of unsealed ghosts. The time course for the loss in transport activity seen in Fig. 5A is comparable (±factor of 2) to that published previously [10] for ghosts incubated at 37°C, but performed under slightly different experimental conditions (see Section 2).

In Fig. 5B we see a shift in the fluorescence spectrum of the DIDS-band 3 after incubation of DIDS-labeled ghosts for 120 h at 37°C. This spectral shift is indicative of a change in the conformation of the band 3 membrane domain, in agreement with previous findings under somewhat different buffer conditions [10]. Taken together, these results establish that a conformational change occurs in the membrane domain of band 3 at 37°C, which correlates with the conformational change in unsealed ghosts associated with a selective increase in plasma immunoglobulin binding to the cytoskeleton.

4. Discussion

The experiments presented in this paper were designed to characterize immunoglobulin binding to membranes from hemolyzed erythrocytes as a function of time and the temperature present during the ghosts' preincubation period. The plasma immunoglobulin binding assay was performed under exactly the same experimental conditions for all of the samples studied. Thus, any changes in immunoglobulin binding capacity reflect a change in the structure of the membrane. The results indicate: (a) that the cytoskeleton is the predominant binding site for naturally occurring autoantibodies present in plasma; (b) that incubation of ghosts at 37°C increases immunoglobulin binding to the cytoskeleton by 2.5-fold, without increasing binding to band 3 or to the other major components contained within the

lipid bilayer; and (c) that this increase in immunoglobulin binding occurs at a rate which is similar to the rate of conformational change in band 3, a major cytoskeletal attachment site within the lipid bilayer.

Comparison of the results in Fig. 1A,C shows that incubation of ghosts at 37°C leads to a large increase in immunoglobulin binding to the inner surface of the erythrocyte membrane (Fig. 1A), without increasing binding at the outer surface (Fig. 1C). The outer membrane surface is probably responsible for the background IGAF488 binding, since Alexa 488 fluorescence is seen in the supernatant fraction, but not in the pellet after solubilization in non-ionic detergent (Fig. 2, Initial (−) plasma). The components of the lipid bilayer which bind background IGAF488 would have interacted with immunoglobulins in vivo, since most of these membrane elements are transmembrane proteins which are exposed to plasma (e.g, band 3). The fact that background IGAF488 binding (no plasma pretreatment) occurs to components contained within the membrane bilayer only, and not to the cytoskeleton (Fig. 2), is consistent with our use of IGAF488 as a specific probe of membrane-bound immunoglobulins.

Treatment of ghosts with plasma specifically increases IGAF488 binding to the cytoskeleton, without significantly increasing the amount of probe bound in the solubilized lipid bilayer fraction (Fig. 2, Initial (−) versus (+) plasma). Incubation at 37°C substantially increases specific opsonization of the cytoskeleton while it remained associated with the bilayer in unsealed ghosts (Fig. 2, 120 h (+) plasma). There was no evidence for band 3 aggregation under our experimental conditions as indicated by the total absence of DIDS fluorescence in the void volume of the column in Fig. 3. SDS-PAGE analysis confirmed the DIDS fluorescence measurements by indicating the absence of band 3 in the void volume of the column.

The observation, in Fig. 2, of increased immunoglobulin binding to the cytoskeleton after incubation of ghosts at 37°C implies that some type of conformational change occurs in ghosts, which exposes autoantibody binding sites on the cytoskeleton. Although this change could be localized to spectrin molecules within the cytoskeleton, the evidence indicates that the entire membrane may be involved in the conformational change which leads to exposure of new cytoskeletal immunoglobulin binding sites. We found that the rate of exposure of cytoskeletal binding sites and the rate of change in the conformation of the membrane domain of band 3 were the same (Fig. 5A). Band 3 is a major cytoskeletal attachment site through binding of the connecting protein known as ankyrin [21]. While further experiments are needed, it is not unreasonable to suppose that the conformational change in the membrane domain of band 3 and the increase in the number of immunoglobulin binding sites on the cytoskeleton are linked. For example, there is clear and ample evidence in the literature that the conformation of the membrane domain of band 3 can influence binding events at the cytoplasmic domain [22,23]. In addition, several laboratories have shown that the conformational stability of the membrane domain is significantly influenced by attachment of the cytoplasmic domain [24,25].

We summarize our results schematically in Fig. 6, within the context of a plausible in vivo model for opsonization of hemolyzed human erythrocytes at 37°C. Fig. 6 shows an intact erythrocyte with band 3 in the native conformation, exposed to the outer surface of the lipid bilayer. The cytoskeleton is labeled with black dots to distinguish it from the bilayer. In step 1, plasma immunoglobulin would gain access to the interior of the cell after an intravascular hemolytic episode, where hemoglobin is released into the plasma and plasma proteins enter the cell. This would lead to initial binding of immunoglobulin to cytoskeletal sites as demonstrated by our results (Figs. 1 and 2). Because this is an in vivo schematic model, we would expect the ghosts to reseal at the physiological salt concentration present in blood [12]. Thus, we show a sealed spherical ghost between steps 2 and 3 in Fig. 6. In our in vitro experiments, ghost resealing was inhibited (see Section 3) in order to allow for quantitation of immunoglobulin binding at the inner surface of the membrane. In step 2 of Fig. 6, band 3 undergoes a conformational change at 37°C [10], which is correlated in time with exposure of additional immunoglobulin binding sites on the cytoskeleton (Fig. 5A). While the exact mechanism for linking these two

changes is not established, testable possibilities can be suggested. The conformational change in band 3 (Fig. 5) could allow it to be more mobile in the plane of the lipid bilayer, consequent to a diminished interaction of the cytoplasmic domain of band 3 with the cytoskeleton [26]. The cytoskeleton could be partially displaced from proximity with the lipid bilayer, which may allow for increased immunoglobulin binding. Such changes could promote the budding of cytoskeleton-free membrane vesicles ([26] and Fig. 6). Evidence in the literature indicates a role for membrane vesicle shedding in erythrocyte homeostasis [27], and it has been suggested that band 3-lipid interactions play a significant role in such a vesiculation process [28]. We envision that over time, this vesiculation process leads to a cytoskeleton which is both 'stripped' of bilayer and heavily opsonized by immunoglobulin molecules (Fig. 2).

Our results seem most relevant to conditions involving intravascular hemolysis [29]. Numerous conditions can cause intravascular hemolysis, such as microangiopathic hemolytic anemia, associated with thrombotic thrombocytopenic purpura. In this latter condition, there is clear evidence for red cell fragmentation which is thought to arise from the interaction of the red cell with fibrin strands in the partially thrombosed microvasculature. Microangiopathic hemolytic anemia is observed in several multisystem disorders that also affect the kidney, including malignant hyperthermia, renal cortical necrosis and other conditions. Patients with systemic lupus erythematosus can have a thrombotic microangiopathic syndrome that includes microangiopathic hemolytic anemia [29]. Fragmentation hemolysis has been found after replacement of aortic and mitral valves with prosthetic devices. Finally, various chemical and physical agents can produce intravascular hemolysis [29]. Serum immunoglobulin binding to the cytoskeleton may be expected to occur in these various conditions.

In summary, the results of this paper establish that the cytoskeleton is the predominant target for plasma immunoglobulin binding to hemolyzed, but otherwise intact human erythrocyte membranes. We suggest that cytoskeletal opsonization occurs as an initial step in the removal of hemolyzed erythrocytes from the circulation.

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