Increased Fluidity of Human Platelet Membranes during Complement-Mediated Immune Platelet Injury

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ABSTRACT Complement appears to be involved in the destruction of platelets in certain clinical disorders, such as quinidine purpura and post-transfusion purpura. In both disorders, the classical complement sequence is activated by antigen-antibody complexes. It has been suggested that the terminal components of the complement sequence insert into the hydrophobic core of cell surface membranes and that this process leads to cell lysis. Fluidity is a fundamental property of lipids within the membrane's hydrophobic core. To examine the interaction of complement with membranes, we investigated the effect of complement activation on the fluidity of human platelet membranes. Complement was fixed to platelets using a post-transfusion purpura antibody, and membrane lipid fluidity was assessed in terms of fluorescence anisotropy using two fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene and 9-(12-anthroyl) stearic acid. Microviscosity, expressed in poise, was derived from the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene.

Post-transfusion purpura antibody plus complement made platelet membranes more fluid as evidenced by a 21% decrease in anisotropy and a 35% decrease in microviscosity of platelets at 37°C, and this was associated with platelet lysis (^{51}Cr release). Complement damage to platelets was accompanied by a 10–15% increase in ΔE, the fusion activation energy for microviscosity, indicating that complement not only decreased membrane microviscosity but also made membrane lipids less ordered. These changes were consistent and rapid, with platelet lysis and the reduction in microviscosity being half-maximal by 6 min. They were prevented by inactivation of complement with heat or with EDTA, and they were not observed when C5-deficient plasma was used as the complement source. Qualitatively similar changes in platelet membrane fluidity were observed when complement was fixed to platelets by a quinidine-dependent anti-platelet antibody rather than by post-transfusion purpura antibody. Post-transfusion purpura antibody plus complement also decreased the microviscosity of isolated platelet membranes. Moreover, the lipids extracted from platelets lysed by complement had a 22% decrease in microviscosity ($P < 0.01$), with no associated changes in the amount of cholesterol relative to phospholipid or in the amounts of the various phospholipids.

These studies demonstrate that lipids within the hydrophobic core of platelet membranes damaged by complement become more fluid, and this is associated with platelet lysis. These findings are consistent with the concept that the insertion of the terminal complement components into the platelet membrane bilayer perturbs lipid-lipid interactions within the membrane's hydrophobic core.

INTRODUCTION

The classical complement sequence (C1-9) is a multimolecular system of plasma glycoproteins which is activated by antigen-antibody complexes. Complement activation may result in the elaboration of biologically active polypeptides (C3a, C5a) that stimulate specialized cell functions, and it may also result in the assembly of a "membrane attack complex" (C5b-9) which damages cell membranes and thereby lyses cells (1).

Human platelets are damaged by complement in vitro through activation of either the classical (2) or

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alternative (3) complement pathways. In a clinical setting, lysis of platelets by complement appears to be responsible for thrombocytopenia in certain diseases, most notably, quinidine or quinine thrombocytopenia (4) and post-transfusion purpura (5). Recent evidence indicates that membrane damage by complement requires the insertion of hydrophobic portions of the membrane attack complex into the hydrophobic core of the cell membrane (6, 7). Fluidity defines a fundamental property of membrane lipids within this core (8).

The fluidity of cell membrane lipids has been evaluated in many types of eukaryote cells using membrane probes of the electron-spin resonance (9) and fluorescent (10) types. From these studies, the general concept has emerged that fluidity of the lipid bilayer is regulated within narrow limits, and if these limits are exceeded, abnormalities in membrane function result (11). The precise mechanism whereby complement insertion disrupts membrane function is unknown. Complement lysis of sheep erythrocytes has been associated with increased fluidity of membrane lipids, as assessed by a spin-labeled phospholipid (12). Therefore, we investigated the effect of complement activation on the fluidity of human platelet membranes. To do this, complement was activated in the presence of human platelets by human anti-platelet antibody from a patient with post-transfusion purpura and from a patient with quinidine thrombocytopenia. Platelet membrane lipid fluidity was assessed in terms of fluorescence anisotropy and microviscosity using the hydrophobic fluorescent probe, 1,6-diphenyl-1,3,5,-hexatriene (DPH).[1]

METHODS

Sources of complement-fixing anti-platelet antibody. Post-transfusion purpura plasma (PTP plasma) was the source of antibody used in most of these studies. It was obtained from a patient whose illness conforms to the classic description of PTP of the PlA1 type (5). Plasma was collected by therapeutic plasmapheresis on a Haemonetics model 30 Cell Separator Blood Processor (Haemonetics Corp., Natick, Mass.), collected into acid-citrate-dextrose (JA-25N Blood Pack, Fenwall Laboratories, Inc., Morton Grove, Ill.), and stored at −70°C until use. PTP plasma caused lysis (^{51}Cr release) in vitro of platelets containing the PlA1 antigen, whereas it failed to lyse PlA1-negative platelets and the patient's own platelets in vitro upon recovery. Several lines of evidence indicated that complement was required for platelet lysis. Platelet lysis did not proceed in a reaction mixture containing gel-filtered platelets and partially purified antibody in the absence of complement and did not proceed in the presence of plasmas deficient in C1 and C2 or selectively deficient in C4.[2] The anti-platelet activity was partially purified by quaternary aminoethyl Sephadex and Sephadex G-200 chromatography and fractionated with monomeric IgG (13). Although the PTP plasma also contained an antibody to HLA-B7, this was not responsible for platelet lysis, since platelets from donors lacking HLA-B7 were still lysed by this plasma. Plasma containing a quinidine-dependent anti-platelet antibody was kindly supplied by Dr. Richard Aster (Milwaukee Blood Center, Milwaukee, Wis.).

Incubation of intact platelets with antibody and complement. The PlA1 antigen is present on platelets of over 98% of the population (5), and the platelets of all donors in these experiments were lysed readily by PTP plasma in the presence of complement. Venous blood was obtained from healthy, fasting volunteers and collected through 19-gauge butterfly needles into plastic syringes and anticoagulated with heparin, 2 U/ml. This concentration of heparin had been shown previously to prevent thrombin generation but support complement-dependent platelet lysis more efficiently than higher concentrations of heparin or conventional anticoagulant concentrations of citrate.[2] All blood processing and incubations were carried out in plastic ware. Platelet-rich plasma was obtained by centrifugation of blood at room temperature for 10 min at 180 *g*. The remaining blood was centrifuged at 21,800 *g* for 20 min to obtain platelet-poor plasma. Platelets were counted (14) and platelet-rich plasma was adjusted with platelet-poor plasma, when necessary, to 3–4 × 10^8 platelets/ml. In certain experiments, platelets were labeled with [^{14}C]serotonin (15) or ^{51}Cr (16) before incubation with antibody and complement.

Incubation mixtures (pH 7.4) contained PTP plasma and an equal volume of platelet-rich plasma, which contained platelets and fresh, heparinized plasma as a complement source. Mixtures were incubated for 1 h at 37°C in a shaking water bath (80 oscillations per min). "Control" incubation mixtures contained the same platelet-rich plasma and an equal volume of autologous or ABO-compatible acid-citrate-dextrose plasma rather than PTP plasma. After incubation, complement activation was stopped by placing the tubes in ice and diluting the reaction mixtures with an isotonic, EDTA-containing platelet buffer ("wash" buffer) (17).

A single experiment was performed to test the effect of PTP antibody on platelet membrane fluidity in the presence of a complement source known to be deficient in C5 (18). Platelets were gel-filtered through Sepharose 2B (19) in modified Tyrode's buffer (20), containing 0.3% crystalline human serum albumin, 0.1% dextrose, and 2 mM $MgCl_2$. They were incubated at 37°C for 1 h, with equal volumes of partially purified PTP antibody and heparinized C5-deficient human plasma. The results were compared with similarly treated platelets incubated with antibody and normal plasma.

Platelets were incubated with quinidine sulfate (2 mM), quinidine-dependent anti-platelet antibody, and complement as described by Aster et al. (2, 21).

Incubation of platelet membranes with antibody and complement. Platelet membranes were isolated by the glycerol lysis method (22), and washed twice at 4°C in the presence of proteolytic inhibitors (23). Where indicated, platelets were also lysed by homogenization (20). Membranes were suspended to a concentration of 500 μg membrane protein per ml in inhibitor-free, modified Tyrode's buffer, pH 7.4, (20). 1-ml membranes were incubated at 37°C with 9 ml PTP plasma and 4.5 ml fresh, heparinized plasma for 1 h, pH 7.4, in a shaking water bath. As a control, membranes were incubated with autologous acid-citrate-dextrose plasma instead of PTP plasma.

Analysis of platelet microviscosity. The fluorescent probe, DPH (1 μM) (Aldrich Chemical Co., Inc., Milwaukee, Wis.), was used to label platelets (1 × 10^8/ml) or isolated platelet

[1] *Abbreviations used in this paper:* AS, 9-(12-anthroyl) stearic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; ΔE, fusion activation energy for microviscosity; PTP, post-transfusion purpura.

[2] Cines, D. B., and A. D. Schreiber. Manuscript in preparation.

membranes (100 μg membrane protein/ml) after each had been washed three times with platelet wash buffer (17). In additional experiments, the lipids of platelets were extracted (24), dessicated under vacuum, dispersed by sonication under nitrogen with the microtip of a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.) (50 W × 2 min) in the original volume of wash buffer, and labeled with DPH. The optimal conditions for labeling platelets with DPH and the instrumentation used to measure fluorescence intensity and fluorescence polarization were previously described (10). Rotational diffusion of DPH within the membrane bilayer was assessed in terms of fluorescence anisotropy and microviscosity (25, 26). The term, membrane fluidity, is used here as synonomous with 1/microviscosity (27).

Excited state lifetimes (t) were estimated from the relative fluorescence intensities at each temperature and from a value of t_0 of 11.4 ns. These corresponded to independent lifetime measurements for DPH in platelets made at 24–25°C using an Ortec photon-counting fluorescence lifetime instrument (Ortec Inc., Oak Ridge, Tenn.) (10). We found that the decay of fluorescence emission could be described in terms of a single exponential.

The anisotropy of a second fluorescent probe of the lipid bilayer, 9-(12-anthroyl) stearic acid (AS), was also studied after platelets were incubated with PTP plasma and complement. Washed platelets (1.6×10^8 ml) were incubated in a suspension of AS (2 μM) at 37°C for 2 h before fluorescence measurements.

Platelet lipid composition. Intact platelets and platelet membranes were washed three times with platelet wash buffer and extracted with 80 vol of isopropanol and chloroform (24). Extracts were freed of nonlipid phosphorous by thrice-washing with 0.05 M KCl (1/5th vol). Platelet (28) and membrane (29) cholesterol and lipid phosphorous content (30) were measured in quadruplicate aliquots. Phospholipids were separated by thin-layer chromatography on silica gel HR with chloroform:methanol:glacial acetic acid:water (50:28:10:5) (31, 32). Spots were visualized by iodine vapor, and the gel was quantitatively recovered for measurement of phosphorous (31). Lipid phosphorous recovery from thin-layer plates ranged from 90 to 96%. Protein was measured by the method of Lowry et al. (33).

Statistics. Values are expressed as the mean ± 1 SEM. The difference between means was assessed by the Student's t test for paired data, computed with a Wang-500 computer (Wang Laboratories, Inc., Lowell, Mass.) equipped with a Wang-500 statistical tape.

RESULTS

Effect of antibody and complement on platelet membrane fluidity. The fluorescence anisotropy and calculated microviscosity of platelet membranes were determined by labeling intact platelets with the fluorescent probe, DPH. This presumably reflects an average anisotropy and microviscosity of the lipids of both surface and internal membranes (10, 34). Unincubated platelets from 14 donors had an average membrane anisotropy of 0.188±0.001 and an average microviscosity of 2.56±0.03 poise at 37°C (Table I). These values were unchanged after a 1-h incubation at 37°C in the presence of complement (fresh, heparinized plasma) but in the absence of antibody (anisotropy = 0.188±0.002; microviscosity = 2.56±0.05 poise). In contrast, platelets incubated with complement (fresh, heparinized plasma) and antibody (PTP plasma) underwent a mean 21% decrease in anisotropy, and this was significant ($P < 0.001$) (Table I). The lifetime of fluorescence emission (t) for DPH in platelets at 37°C was 7.1 ns, and this was unaffected by incubation with antibody and complement. As a result of the decrease in anisotropy with no change in fluorescence lifetime, the calculated microviscosity at 37°C of platelets incubated with antibody and complement decreased by 35% (Table I). This decrease was consistent and significant ($P < 0.001$) (Fig. 1). The complement-induced change in microviscosity occurred rapidly, and was half-maximal by 6 min (Fig. 2). Inactivation of complement by heating (56°C for 45 min) or preventing its activation with EDTA (10 mM) did not prevent antibody (IgG) binding to platelets[2] but did prevent changes in microviscosity (2.55±0.09 poise).

Changes in membrane microviscosity were associated with platelet lysis. Incubation of platelets with PTP plasma and complement resulted in an average 34% release of platelet ^{51}Cr (range, 10–68%). The time-courses of ^{51}Cr release and the change in plate-

TABLE I

Complement-Induced Changes in Fluorescence Anisotropy and Microviscosity of Platelets, Isolated Membranes, and Sonicated Lipids of Platelets labeled with DPH

	Anisotropy, 37°C		Microviscosity, 37°C (poise)	
	Normal	Complement-damaged	Normal	Complement-damaged
Platelets (14)*	0.188±0.001	0.148±0.005	2.56±0.03	1.66±0.09
Platelet membranes (4)	0.184±0.002	0.170±0.004	2.44±0.04	2.09±0.07
Platelet lipids (6)	0.158±0.004	0.136±0.004	1.82±0.08	1.42±0.06

* Numbers in parentheses indicate number of experiments.

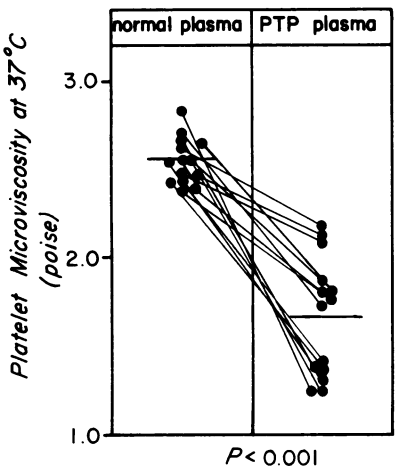

FIGURE 1 The effect of PTP plasma and complement on platelet microviscosity. Horizontal bars represent the means of each group. Fine lines connect platelets incubated in normal plasma with the same platelets incubated in PTP plasma.

let microviscosity were similar. ^{51}Cr release from platelets did not occur with heat-inactivated plasma or in the presence of EDTA. No change in platelet microviscosity was observed when platelets were lysed by glycerol-loading or by homogenization. However, disruption of platelets with Triton X-100 (0.003 vol/100 ml) resulted in 84% ^{51}Cr release and an 11% decrease in microviscosity ($P < 0.01$). Thus, the changes in microviscosity caused by antibody and complement were neither a universal concomitant of membrane lysis nor were they unique to complement lysis.

To determine whether the effect of antibody and

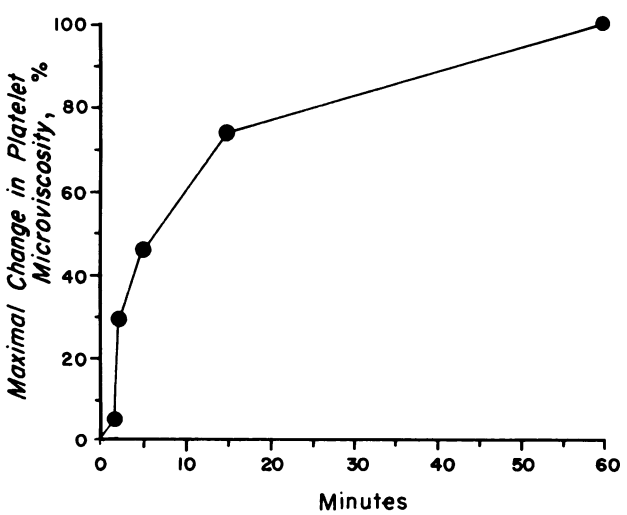

FIGURE 2 Time-course of changes in platelet microviscosity induced by PTP plasma and complement.

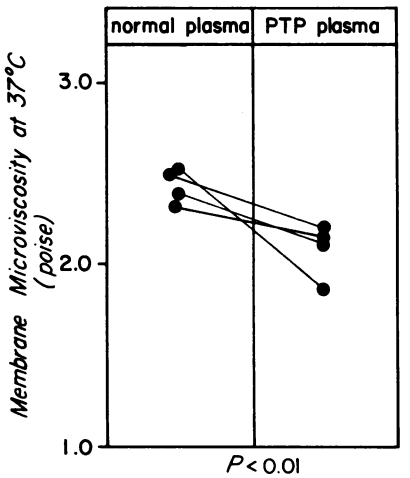

FIGURE 3 The effect of PTP plasma and complement on the microviscosity of isolated platelet membranes.

complement on lipid microviscosity was indeed a result of changes in platelet surface or surface-connecting membranes, the membrane fraction of platelets was separated from platelet granules. Incubation of isolated platelet membranes with PTP plasma and complement consistently resulted in a decrease in membrane microviscosity ($P < 0.01$) (Fig. 3; Table I). Complement-induced membrane damage resulted not only in the bilayer of isolated membranes becoming more fluid, but the lipids extracted from complement-damaged platelets were more fluid as well. Thus, platelets were lysed with PTP plasma and complement and their lipids were extracted, dispersed by sonication, and labeled with DPH. The microviscosity of the extracted lipids was 22% lower than the lipids of normal platelets (Fig. 4; Table I). The microviscosity of lipids of platelets incubated with complement but in the absence of antibody was 1.81 ± 0.05 poise and was comparable to that of normal platelet lipids (Table I).

The relationship between temperature and microviscosity in platelets before and after complement-mediated membrane damage is shown in Fig. 5. Characteristic of biomembranes, a plot of the logarithm of platelet microviscosity against the reciprocal of the absolute temperature was linear, both for normal and complement-damaged platelets. The microviscosity of complement-damaged platelets was lower than normal over the temperature range studied (6°–49°C). The slope of a line on this plot is a measure of ΔE, the fusion activation energy for microviscosity. This is an expression which characterizes the degree of order within lipid-lipid interactions, a higher value for ΔE indicating less order. Complement-damaged platelets exhibited a ΔE 10–15% higher

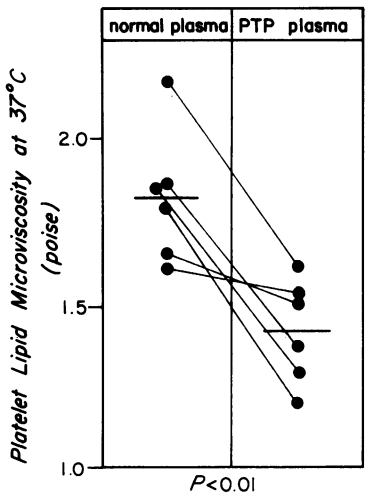

FIGURE 4 The microviscosity of lipids extracted from platelets previously incubated with PTP plasma and complement.

than normal (Fig. 5). Thus complement had a dual effect on platelet membrane lipids: it decreased their microviscosity and it made them less ordered.

PTP antibody was not the only complement-fixing anti-platelet antibody to decrease membrane viscosity. A quinidine-dependent anti-platelet antibody plus complement caused a 12% decrease in platelet microviscosity, and this was associated with the release of 44% platelet ^{51}Cr. Moreover, the effect of complement on platelet membrane fluidity was demonstrable using a second hydrophobic fluorescent probe, AS. In two experiments, the fluorescence anisotropy of AS in platelets decreased 11% after incubation of these platelets with PTP plasma and complement.

Mechanism of the complement effect on platelet membrane fluidity. These studies demonstrate that the microenvironment of DPH in intact platelets, isolated platelet membranes, and extracted platelet lipids becomes markedly more fluid as a result of

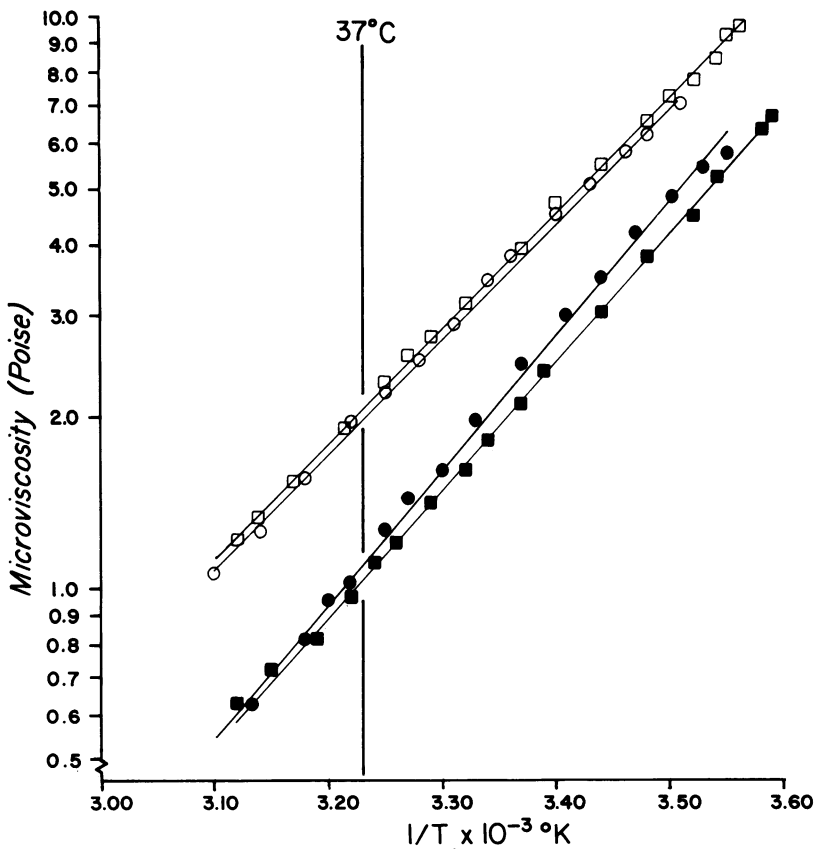

FIGURE 5 The effect of PTP plasma and complement on platelet microviscosity and the fusion activation energy for microviscosity, ΔE. The white circles and squares depict the relationship between microviscosity (plotted on a logarithmic scale) and the reciprocal of the absolute temperature (plotted on an arithmetic scale) in normal platelets from two different donors. The black circles and squares depict the same platelets incubated with PTP plasma and complement. The slopes of these lines are used to calculate ΔE, which was 9.3 and 9.4 kcal/mol for normal platelets, and 10.2 and 10.8 kcal/mol for complement-damaged platelets.

complement-mediated membrane damage. They do not identify the precise locus of the classical complement sequence where changes in membrane fluidity are initiated. However, microviscosity was unaffected when gel-filtered platelets were incubated with the IgG fraction of PTP plasma and C5-deficient plasma as the complement source. This indicates that changes in microviscosity require activation at least through C5. In addition, when platelet microviscosity, platelet secretion (measured by [^{14}C]serotonin release), and lysis (^{51}Cr release) were measured after incubation of platelets with complement and serial twofold dilutions of PTP plasma, decreases in membrane microviscosity were observed only at high antibody concentrations. Cell lysis by complement is believed to occur only after the attachment of C8 to the C5b, 6, 7 complex (35). Platelet lysis was observed only at the same high antibody concentrations at which changes in membrane microviscosity were observed. In contrast, low relative concentrations of antibody, which release [^{14}C]serotonin only in the presence of complement but do not cause platelet lysis,[2] did not affect platelet membrane microviscosity. Taken together, these data are consistent with the observations of Hammer and co-workers that the terminal complement components insert into the membrane lipid bilayer (6), and they suggest that complement insertion results in fluidization of the platelet membrane lipid bilayer.

Complement could make platelet membranes more fluid by perturbing normal lipid-protein interactions, lipid-lipid interactions, or lipid composition. Although an effect on lipid-protein interactions was not excluded, the fact that sonicated lipids from complement-damaged platelets were more fluid than normal (Fig. 4) suggests a physical rearrangement or compositional change within the lipids themselves. However, we were unable to detect any compositional changes in the lipids of isolated platelet membranes incubated with antibody and complement. In five experiments, there was no change in the relative distribution of the major phospholipid classes, including lecithin, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and lysolecithin. The amount of cholesterol relative to phospholipid in membranes damaged by antibody and complement (0.55 ± 0.05) was similar to that of normal membranes (0.56 ± 0.03).

DISCUSSION

These studies demonstrate that activation of complement by human antiplatelet antibody resulted in a rapid and marked increase in the fluidity of human platelet membrane lipids and that this was associated with platelet lysis. These changes were demonstrable whether complement was activated by PTP antibody or by a quinidine-dependent antibody. That platelet membranes became less ordered and more fluid was apparent whether assessed by direct measurements of fluorescence anisotropy with DPH or AS or by calculating membrane microviscosity and ΔE for microviscosity with DPH.

Although these data may be relevant to the mechanism of complement-induced membrane injury and cell lysis, several factors must be considered and controlled for when applying fluorescence polarization techniques to the study of biomembranes. Fluorescence measurements derived from intact platelets may not be indicative of probe rotation within the surface membrane alone, particularly since DPH may be distributed throughout surface and internal membranes of the platelet (10). Studies in other complex cells have also demonstrated a generalized distribution for DPH (34, 36). Therefore, we separated the platelet "membrane fraction" from granule membranes, and demonstrated that isolated membranes were indeed more fluid after their interaction with antibody and complement.

Hydrophobic probes, such as DPH, label hydrophobic proteins as well as lipids. Thus it is possible that complement components which insert into the platelet membrane constitute a new "microenvironment" for DPH, thereby confounding interpretation of the fluorescence data with respect to rotational diffusion of the probe within the membrane lipid bilayer. This theoretical problem appears unlikely in the present study since the complement-induced changes in fluidity were also observed in sonicated lipids extracted from platelets.

Biomembranes obviously display a greater complexity and heterogeneity of lipid-protein and lipid-lipid interactions compared to pure phospholipid dispersions (37). The assessment of "microviscosity" by analysis of the rotational diffusion of DPH in biomembrane lipids by relating it to the fluorescence polarization and lifetime of the probe in paraffin oils is only semi-quantitative. Although it is useful to compare values of microviscosity in control platelets with values in the same platelets after some perturbation (i.e. complement), it may not be proper to consider the microviscosity values in units of poise as absolutely comparable to macroscopic systems. Nonetheless, our studies of human platelets with fluorescent probes are comparable to the spin-label studies of sheep erythrocyte membranes which demonstrate fluidization of the lipid bilayer by complement (12).

Several factors are known to decrease membrane anisotropy and microviscosity. Interaction of membrane lipids (boundary lipids) with membrane proteins may contribute normally to the microviscosity value since extraction of lipids from membrane proteins results in a decrease in lipid microviscosity

(38, 39). This was true in platelets as well; however the extracted lipids of platelets damaged by complement still were more fluid than normal platelet lipids (Fig. 4). These data suggest that some change in lipid composition had occurred. Theoretically, platelet membrane lipids could be made more fluid by a decrease in the amount of membrane cholesterol relative to phospholipid (10) or by a decrease in the amount of lecithin relative to sphingomyelin (40). However, complement lysis was associated with no detectable change in the amount of cholesterol relative to phospholipid or in the relative distribution of the major phospholipid classes in platelet membranes. The precise mechanism whereby complement activation results in a greater fluidity of platelet membrane lipids remains unknown.

The changes in platelet membrane fluidity during complement lysis are not a general result of platelet disruption since glycerol lysis or homogenization did not affect membrane microviscosity. It has been suggested that the increased fluidity of the cell membrane by complement may be the proximate cause of membrane lysis (12). Indeed, local anesthetics increase membrane fluidity at low concentrations (41) and cause membrane lysis at higher concentrations (42). Moreover, Kinsky has suggested that the lytic action of complement resembles that of a detergent (43) and, in the present study, the detergent, Triton X-100 increased platelet membrane fluidity as it disrupted platelets. On the other hand, Mayer has theorized that the increased permeability of complement-damaged membranes might result from the formation of a hydrophobic channel formed by one or more of the terminal complement components (44). Thus, whether the fluidity changes induced by complement are causative of lysis or merely a concomitant of the lytic process remains unknown.

An enzymatic degradation of phospholipids is another potential mechanism by which complement damages membranes. Complement activation can cause leakage from artificial lipid dispersions (liposomes) in the absence of protein (43) and lysolecithin, the product of phospholipase A activity, is a detergent (45). However, careful studies with liposomes (43, 46) and *Acholeplasma laidlawii* (47) have failed to demonstrate phospholipase activity as a proximate cause of membrane lysis by complement. In addition, we found no evidence for lysolecithin generation in platelets during complement damage. These negative data make enzymatic degradation of phospholipids unlikely, but they do not unequivocally exclude this possibility, as they are limited by the sensitivity of the analytical techniques employed.

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