PLATELET-MEDIATED CYTOTOXICITY
Role of Antibody and C3, and Localization of the Cytotoxic System
in Membranes

BY SHERI SLEZAK, DAVID E. SYMER, AND HYUN S. SHIN

From the Department of Molecular Biology and Genetics, Subdepartment of Immunology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Antibody-dependent cell-mediated cytotoxicity (ADCC) is believed to play an important role in the immunologic destruction of neoplastic cells, parasites, virally infected cells, and foreign grafts, as well as in the pathogenesis of autoimmune diseases (1-4). While the participation of macrophages, lymphocytes, and granulocytes in this process is widely recognized, the involvement of platelets has only recently been appreciated.

The indication that platelets may mediate ADCC in vivo came with the observation in irradiated mice that the growth of grafted lymphomas was suppressed in the presence of platelets and anti-tumor antibody (5-7). Independently, the in vitro lysis of antibody-sensitized chicken erythrocytes and of human erythrocytes and mastocytoma cells was briefly reported (2, 8). In an extensive quantitative study, Soper et al. (9) showed that lysis of antibody-sensitized SRBC occurred in platelet-rich, C5-deficient plasma. The role of platelets and C3 was demonstrated by the lack of lysis in C5-deficient platelet-poor plasma, and in C3-depleted platelet-rich plasma (9). Others have reported (10) that human platelets lyse cells from a human renal cell carcinoma line and a melanoma cell line without added antibody. Platelet-mediated destruction of schistosomula in rats has been reported to involve IgE (11) and C-reactive protein (12).

Recently, much progress has been made in our understanding of the mechanism of effector cell activation by antibody and complement through the structural and functional characterization of receptors for these molecules (13-19). Moreover, much work has been done to elucidate mechanisms of cytotoxicity; these include the generation and action of toxic oxygen metabolites (20), pore-forming proteins (21-23), and neutral proteases (24-27). In an effort to study the mechanism of platelet-mediated cytotoxicity at a molecular level, we have developed a plasma-free system where platelets lyse washed SRBC bearing
defined molecular components. Our results demonstrate that antibody and C3, in close proximity on target cell surfaces, synergistically activate the platelet for cytotoxicity. Furthermore, our results suggest that the cytotoxic system capable of both recognition and lysis resides in membranes.

Materials and Methods

**Animals.** 5–10-wk-old male AKR/J and 8-wk-old female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Antibodies.** Pooled rabbit polyclonal anti-SRBC antibody was prepared as outlined by Kabat and Mayer (28), and fractionated rabbit polyclonal IgG and IgM anti-SRBC antibodies were purchased from Cordis Laboratories Inc. (Miami, FL). The hemolytic titers of these antibody preparations were determined as described (28). Mouse monoclonal IgG1 and IgG2b anti-SRBC, and IgE anti-DNP antibodies were from Pel-Freez Biologicals (Rogers, AR). Hybridomas that produce mouse monoclonal IgG2a, IgG2b, IgG3, and IgM anti-SRBC antibodies were obtained from American Type Culture Collection (Rockville, MD) through the courtesy of Dr. William Raschke, La Jolla Cancer Research Foundation (La Jolla, CA). Each of these antibodies was collected both from culture supernatants and from ascites fluid that formed in BALB/cJ mice pretreated with 0.75 ml pristane (Aldrich Chemical Co., Milwaukee, WI) 3–20 d before injection of hybridoma. Goat anti-mouse κ chain antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), goat anti-mouse C3 antibody was from Cappel Laboratories (Malvern, PA), and rabbit-anti-goat IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

**Buffers.** Tyrode’s solution, pH 7.3, contains 8 g NaCl, 0.2 g KCl, 0.255 g CaCl₂, 0.1 g MgCl₂·6H₂O, 0.0435 g NaH₂PO₄, 1 g glucose, and 1 g NaHCO₃ per liter. To make Tyrode’s gel-EDTA, 1 g gelatin and 40 ml of 0.1 M EDTA stock solution were added per liter of Tyrode’s solution. This solution was filtered through a 0.45-μm filter (Nalge Co., Rochester, NY) and stored at 4°. Veronal-buffered saline with gelatin (VBSG) pH 7.4, contains 8.3 g NaCl, 1.0 g sodium barbital, 16.7 mg CaCl₂, 0.20 g MgCl₂·6H₂O, and 1.0 g gelatin per liter. Dextrose-containing VBSG (DVBSG) consists of one part VBSG, and one part 5% glucose solution with 0.1% gelatin. EDTA buffer, 0.01 M, was made by adding one part 0.1 M stock EDTA solution to nine parts VBSG. Cacodylate buffer, pH 6.9, consists of 38.6 g cacodylic acid per liter. HBSS and RPMI 1640 were from Whittaker M.A. Bioproducts (Walkersville, MD) and FCS was from HyClone Laboratories (Logan, UT). TCM-10 medium was made by adding one part FCS to nine parts RPMI 1640.

**Reagents and Plasticware.** All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except as noted. Plastic Eppendorf tubes were from Brinkmann Instruments Co. (Westbury, NY), plastic test tubes from Falcon Labware (Oxnard, CA), and 96-well round-bottomed microtiter plates and plastic pipette tips were from Costar (Cambridge, MA).

**Platelets and C5-deficient Plasma.** Mice were anesthetized by inhalation of methoxyflurane (Abbott Laboratories, N. Chicago, IL) and bled by cardiac puncture using a heparinized 3-ml plastic syringe fitted with a 25-gauge needle. Final concentration of heparin (Ivenex, Chagrin Falls, OH) was 15 U/ml blood. Whole blood was pooled into 3-ml plastic test tubes and centrifuged at 90 g for 10 min. We used plasticware to avoid agglutination. The resulting platelet-rich plasma (PRP) was spun at 6500 g for 20 min at 10° C. The supernatant, platelet-poor plasma (PPP), was removed and used as source of complement, while the platelet pellet was resuspended in 700 μl Tyrode-gel-EDTA and centrifuged as above. The supernatant was removed and the washed pellet was resuspended to original volume, giving a solution of ~10⁷–10⁸ platelets/ml, as counted in a hemacytometer. There was <0.1% contamination with nucleated cells.

To disrupt platelets, they were sonicated three times on ice for 10 s pulses with a Sonifier Cell Disruptor (No. W140; Ultrasonics, Inc., Plainview, NY), used at setting 5. The sonicate was centrifuged at 12,000 g for 3 min and the pellet was brought to original volume in Tyrode’s-gel-EDTA. Both the supernatant and the resuspended platelets were
assayed for cytolytic activity. In some experiments, sonicated platelets were further disrupted by resuspending pellets in distilled water and incubating for 10 min at room temp (29). These water-shocked sonicated platelets were centrifuged at 12,000 g for 3 min and suspended in Tyrode's-gel-EDTA. Freezing and thawing consisted of placing platelets in an ethanol–dry ice bath for 15 min, followed by thawing in a 37°C water bath for 15 min. This was repeated twice for a total of three cycles. The whole lysate as well as separated pellet and supernatant components were assayed for cytolytic activity.

**Erythrocyte Targets.** SRBC were obtained from a local dealer at 2-wk intervals and stored at 4°C in citrate-dextrose. They were washed in 0.01 M EDTA and then twice in VBSG. An aliquot of 100 µl packed SRBC was labeled with 300 µCi 51Cr in a sterile saline preparation (New England Nuclear, Boston, MA; or Amersham Corp., Arlington Heights, IL), in a shaking, 37°C water bath for 1 h. The cells were centrifuged at 125 g for 5 min, washed three times in saline, resuspended in 9.9 ml DVBSG at 10⁸ cells/ml, and stored at 4°C for use within 1 wk.

To prepare antibody-sensitized erythrocytes (EA), 0.1 ml of 51Cr-labeled SRBC stock was mixed with 0.1 ml antibody in VBSG and incubated with occasional agitation for 60 min at 30°C. To sensitize erythrocytes with both antibody and complement (EAC ~ 3b), 0.2 ml fresh C5-deficient PPP or serum was added to SRBC and antibody, and incubated for 60 min. Cells were then washed twice and resuspended at 10⁷ cells/ml. Unless mentioned otherwise, heat-inactivated polyclonal rabbit anti-SRBC was used in preparing EA or EAC ~ 3b as described (28).

To hapten derivatize erythrocytes so that anti-DNP monoclonal IgE antibody could be tested, 0.3 ml 51Cr-labeled SRBC at 10⁸ cells/ml were centrifuged at 125 g for 5 min and the supernatant was removed. The cells were resuspended in 3.5 ml of 0.28 M cacodylic acid, pH 6.9, containing 15 mg of 2,4,6-trinitrobenzene sulfonic acid. After a 10-min incubation at room temperature, cells were washed three times in VBSG.

**Cytotoxicity Assay.** Serial dilutions of platelets in 25 µl Tyrode's-gel-EDTA were made in round-bottomed microtitre plates. Equal volumes of target cells at 10⁶ cells/ml were added, and 4°C plates were incubated at 37°C for 90 min with continuous agitation to keep target cells in suspension (microorbital shaker, speed 6; Bellco Glass, Inc., Vineland, NJ). After incubation, 150 µl ice-cold Tyrode's-gel-EDTA was added to each well and the plates were agitated for 15 s. Plates were centrifuged at 700 g for 10 min at 4°C and 100 µl supernatant was assayed for 51Cr release in a 4000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). The maximum number of counts was determined with 12.5 µl of labeled target cells.

When inhibitors were tested, they were added in 10-µl volumes, as were control buffer aliquots. The final incubation volume was 60 µl, and after incubation, 140 µl of Tyrode's-gel-EDTA was added. As before, 100 µl of supernatant was assayed for chromium release.

**Quantitative Analysis of Data.** In Figs. 2 and 3, the extent of target cell destruction was calculated as percent lysis = 100 x (cpm sample)/(cpm maximum). In all other cases, we calculated it as percent specific lysis = 100 x [(cpm sample) - (cpm spontaneous)]/(cpm maximum) - (cpm spontaneous)]. All points shown are means of replicate experimental trials. For duplicate values, the range was determined, and for other replicates, the standard deviation was calculated. Error bars are used to indicate those deviations from the mean that exceeded 5%.

**Preparation of Cobra Venom Factor (CVF)-treated Plasma.** 5 U of CVF obtained from Cordis Laboratories Inc. were injected intraperitoneally 18 h and 1 h before AKR/J mice were killed. PPP was obtained as described above for untreated PPP.

**Radioimmunoassay.** Unlabeled SRBC, antibody-sensitized SRBC, or antibody and complement–sensitized SRBC were brought to 10⁸ cells/ml in RIA buffer, a mixture of VBSG and an equal volume of TCM-10, and put in 100-µl aliquots in siliconized glass tubes (VWR Scientific Div., San Francisco, CA). The cells were centrifuged at 700 g at 20°C, for 5 min, and the resulting pellet was resuspended with 1 ml of a mixture of FCS and an equal volume of HBSS. Resuspended SRBC were layered over 0.5 ml RIA buffer and centrifuged as before. The pellet was resuspended in 0.1 ml RIA buffer.

Anti–mouse IgG, anti–κ chain, or anti-C3 antibodies were iodinated (125I; Amersham
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FIGURE 1. Platelet-mediated lysis in plasma-free system. (■) Lysis of EAC ~ 3b by washed platelets in Tyrode’s solution containing 4 mM EDTA and 0.1% gelatin. (□) For comparison, EA was incubated in platelet-rich plasma. Lysis of EA incubated in comparable concentrations of C5-deficient, platelet-poor plasma was <10% (data not shown). Each point represents the mean of duplicate experiments. For definition of error bars, see Materials and Methods.

Corp.) by the chloramine T method (30) and were added in 0.1-ml aliquots. After incubation with occasional mixing for 1 h at room temperature, the cells were centrifuged and the pellet was washed as before. The number of cpm remaining in the washed pellet was determined in a 4000 gamma counter (Beckman Instruments, Inc.).

Marker Assays. To determine lactate dehydrogenase (LDH) activity, 0.4-ml fractions of disrupted platelets were added to an equal volume of NADH (0.2 mg/ml in 0.1 M Na3PO4, pH 7.2). After incubation for 20 min at room temperature, 0.2 ml of pyruvic acid (7 mg/10 ml in 0.1 M Na3PO4, pH 7.2) was added, and the OD450 was immediately recorded for 1 min. As a control, an equal number of intact platelets were completely lysed by incubating in mellitin solution, 2 mg/ml, for 30 min at 37°C, and spun for 5 min. The supernatant was assayed as before to determine total LDH release.

The activity of N-acetyl-B-D-glucosaminidase was measured as follows: 0.1-ml fractions of disrupted platelets were added to the mixture of 0.8 ml of 0.05 M sodium citrate buffer, pH 4.5, and 0.1 ml of 25 mM p-nitrophenyl N-acetyl B-D-glucosaminide and were incubated for 30 min at 37°C. The reaction was stopped with a 1-ml solution of 1 M NaOH and 0.5 M glycine, and OD450 was determined.

1 nm of [3H]serotonin (New England Nuclear) at 29.5 Ci/mmol was incubated with platelets (108/ml) at 37°C for 30 min (31). Platelets were extensively washed before disruption, and the serotonin activities remaining in resulting fractions were determined in a scintillation counter (model LS 1800; Beckman Instruments, Inc.).

Electron Microscopy. Frozen and thawed, sonicated, sonicated and water-shocked, and intact control platelets were prepared as described above. All preparations were pelleted at 12,000 g for 3 min, supernatants were carefully removed, and pellets were fixed in freshly made 0.21 M cacodylate buffer, pH 7.4, containing 4% glutaraldehyde for 2 h at 25°C. The pellets were washed three times with ice-cold 0.21 M cacodylate buffer containing 7.5% sucrose. The samples were further fixed in 1% osmium tetroxide for 60 min at 4°C, dehydrated in a series of graded alcohols, and embedded in epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections, 80 nm thick, were stained with uranyl acetate and lead citrate (Electron Microscopy Sciences). Sections were then examined with a JEOL 10 CX microscope.

Results

Plasma-free System. To study the mechanisms of platelet-mediated lysis, we first developed a system wherein washed platelets could lyse SRBC targets sensitized with antibody and complement in a medium free of plasma. We designate these targets as EAC ~ 3b. As shown in Fig. 1, platelets were able to lyse EAC ~ 3b in Tyrode’s solution containing 0.1% gelatin and 4 mM EDTA. For comparison, we also measured the lysis of EA in C5-deficient platelet-rich plasma, serially diluted in Tyrode’s solution containing 0.1% gelatin, but no EDTA. At equivalent concentrations of platelets, lysis in our plasma-free system was similar to that in the plasma-rich system.

We used the designation EAC ~ 3b even though the active complement fragment may be some cleavage product(s) of C3 other than C3b.
FIGURE 2. Effect of Ca\(^{++}\), albumin, and gelatin in Tyrode's solution. (a) Tyrode's solution with 0.1% gelatin contained 2 mM Ca\(^{++}\), no Ca\(^{++}\), or 4 mM EDTA; (b) Tyrode's solution with 4 mM EDTA contained no gelatin, 0.1% human serum albumin, or 0.1% gelatin. Lysis of EAC\(^{-3b}\) in solutions containing no platelets (open bar), platelets at 1.25 x 10\(^7\)/ml (hatched bar), or 5 x 10\(^7\)/ml (solid bar). Each bar represents the mean of two experiments.

FIGURE 3. Kinetics of platelet-mediated lysis. Lysis of EAC\(^{-3b}\) was determined at various times as indicated. Platelet concentrations were 8 x 10\(^7\)/ml (■); 4 x 10\(^7\)/ml (□); 2 x 10\(^7\)/ml (▲); and 1 x 10\(^7\)/ml (▲). In control (△) no platelets were added. Each point is the mean of three experiments.

We initially used simple Tyrode's solution without EDTA or gelatin, and observed high spontaneous lysis and frequent agglutination of target EAC\(^{-3b}\) in this solution. Elimination of calcium from Tyrode's solution (Fig. 2a) and addition of small amounts of protein such as gelatin or albumin (Fig. 2b) resolved these problems. Therefore, unless mentioned otherwise, in all our experiments we used Tyrode's solution containing 4 mM EDTA and 0.1% gelatin (Tyrode's-gel-EDTA).

Kinetics and Dose Response of the Lytic Reaction. The kinetics of the lytic reaction was examined at several concentrations of platelets. As shown in Fig. 3, a significant lag period preceded lysis, plateau levels were reached by 120 min, and the extent of lysis depended on the concentration of platelets. In other experiments (data not shown), the lag period was shorter and plateau levels were attained sooner, e.g., by 60 min. For routine assay of platelet-mediated lysis, reactions were terminated after 90 min of incubation. When lysis of targets is plotted as a function of platelet concentration, the dose-response curve is entirely concave to the abscissa, as shown in Fig. 4. In only 2 of 15 experiments, the dose-response curves were not concave to the abscissa (e.g., Fig. 1). Because of this occasional variability, additional experiments were performed so that lysis at several low platelet concentrations could be examined more thoroughly. The
result is shown in the inset of Fig. 4. Again, as in most of our experiments, the
dose-response curve is entirely concave to the abscissa. These results indicate
that the process of platelet-mediated lysis is a one-hit reaction, where a fruitful
interaction between one platelet and one target cell leads to lysis. If all platelets
have functional lytic capacity under the assay conditions used, one would expect
that, at a platelet-to-target cell ratio of one-to-one, lysis would be 63% based on
the Poisson distribution. Results from 15 experiments indicate that in one case
lysis failed to reach 63%, even at the highest platelet-to-target ratio that was
tested, which was 5 to 1. In the remaining cases, ratios ranged between 0.5 and
7; most of them clustered between 0.5 and 2, giving an average ratio of 1.3.

Effect of Temperature on Lytic Reaction. The effect of temperature on the
kinetics of lysis was also investigated. As shown in Fig. 5, the rate of lysis increased
as temperature was raised. Because of difficulty in determining the initial reaction
velocity, the extent of lysis at 60-min time points was analyzed as a function of
temperature. We calculated $Z$ values, defined to be the average number of lethal
platelet interactions per target cell (32), where $Z = -\ln (1 - \text{fraction of specific
lysis})$. Changing temperature from 4° to 22°C increased lysis from 6 to 25%,
and $Z$ values from 0.06 to 0.29. Raising temperature from 22° to 37°C increased
lysis from 25 to 62%, and $Z$ values from 0.29 to 0.97. The $Q_{10}$ values calculated
from changes in $Z$ values are therefore 2.4 in going from 4° to 22°C, and 2.2
for the change from 22° to 37°C. From another experiment of similar design,
$Q_{10}$ is 1.3 (4° to 22°C) and 2.1 (22° to 37°C). The average $Q_{10}$ is 2.0.
Role of Antibody and Complement. We investigated whether or not sensitization of targets with antibody alone would be sufficient to induce platelet-mediated lysis, and what role complement might play in the reaction.

We first examined the activity of mouse mAbs of different isotypes. All of the antibodies used, namely IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE, had κ light chains. For comparative studies shown in Fig. 6a, SRBC were sensitized with antibodies of the different isotypes to generate comparable amounts of cell-bound antibody, as measured by the binding of 125I-labeled anti-κ chain antibody. We observed 21% lysis of targets sensitized with IgG2a alone, and <5% with other isotypes alone. Even with 30 times more cell-bound IgG2a than that shown in Fig. 6a, lysis did not exceed 21%. The low degree of lysis of SRBC sensitized with IgM, IgG3, or IgE, as shown in Fig. 6a, was not seen in several other experiments.

In contrast, when antibody-sensitized target cells were treated with C5-deficient mouse plasma, those with isotypes IgG2b or IgG3 were now lysed by platelets, and lysis of target cells sensitized with IgG2a was enhanced. Isotypes IgG1, IgM, and IgE were inactive with or without added complement even with 20 times more cell-bound antibody. The low-level lysis of IgM- or IgE-sensitized EAC-3b targets, i.e. 5–7% in Fig. 6a, was not seen in several other experiments. Thus, among the complement-activating isotypes, namely IgM, IgG2a, IgG2b, and IgG3, only IgM was inactive. Next, the role of C3 in platelet-mediated lysis was evaluated. The platelet-mediated lysis of control target cells sensitized with monoclonal IgG2b and treated with C5-deficient plasma was 63% at platelet concentrations of 5 × 10^7/ml, whereas treatment with plasma that had been C3-depleted by CVF resulted in no lysis even when platelet concentration was increased to 10^8/ml (data not shown). The critical role of C3 in the lytic process is therefore implicated.

We examined the activity of polyclonal antibodies of IgM and IgG isotypes, used at comparable hemolytic titers. As shown in Fig. 6b, 25% of targets treated with polyclonal IgG alone were lysed, and 99% of targets were lysed in the presence of complement. Under comparable experimental conditions, lysis of target cells sensitized with IgG alone ranged between 0 and 32% in 12 cases, and only in one case did lysis reach 62%. Further treatment with C5-deficient plasma, but not C3-depleted plasma, consistently increased the degree of lysis.
Target cells treated with polyclonal IgM, with or without C5-deficient plasma, were not lysed by platelets (data not shown). Hence these results are consistent with those obtained with mAbs.

Spatial Relationship between Cell-bound IgG and C3 Fragment Triggering Platelet-mediated Lysis. The studies described in the preceding section indicated a requirement for activated cell-bound C3, presumably C3b. We investigated whether or not the proximity of cell-bound IgG and C3b affects platelet-mediated lysis. For this purpose, we used mouse monoclonal IgG2b and IgM. Cell-bound IgG2b, together with C3b, induced platelet-mediated lysis, whereas mouse monoclonal IgM generated cell-bound C3b but failed to induce lysis. As shown in Table 1, more IgG2b than IgM was needed to generate comparable amounts of cell-bound C3b on SRBC. These target cells are designated as ElgG2bC~3b and ElgMC~3b, respectively. We sensitized aliquots of ElgMC~3b with IgG2b. This cell preparation is designated as ElgMC~3b + IgG2b. Only ElgG2bC~3b and not ElgMC~3b + IgG2b was lysed, despite the fact that ElgMC~3b + IgG2b had comparable amounts of cell-bound C3b and IgG2b. It is possible that when complement is activated by appropriate cell-bound antibodies, C3b molecules are deposited near antibody molecules and only these C3b can activate platelets in cooperation with the appropriate isotypes of antibody. When washed ElgMC~3b is treated with IgG2b, the sites where freshly added IgG2b bind may be too far from the already-bound C3b molecules to induce lysis.

Lack of Effect of Protease Inhibitors on Lytic Activity. Proteases have been implicated in the cytotoxic reactions mediated by macrophages, neutrophils, and cytolytic T cells (24–27). We tested inhibitors of proteases that included leupeptin, tosyl-l-lysine chloromethyl ketone (TLCK), PMSF, aprotinin, and several trypsin inhibitors. As seen in Fig. 7, there was no significant inhibition of lysis by 0.2 mM PMSF or 0.2 mM TLCK. Similarly, 1 mM leupeptin had no inhibitory
Effect. We observed no significant inhibitory activity with 1 mM aprotinin, 0.75 mM soybean trypsin inhibitor, 1 mM lima bean trypsin inhibitor, or 1 mM chicken egg white ovomucoids types II-0 or III-0 (data not shown). The concentrations of inhibitors and cells used here are similar to those used in other cellular systems, such as neutrophils and macrophages (24, 25). Because of the small size of platelets compared with leukocytes, platelets were exposed to greater amounts of inhibitors on a per cell mass basis.

**Evaluation of the Role of Toxic Oxygen Metabolites.** The toxic metabolites of oxygen have been shown to cause target cell lysis by other effectors (20). Superoxide dismutase, catalase, and mannitol did not affect platelet-mediated lysis (Fig. 8, a and b). Platelets preincubated in glucose-free medium or cytochal-
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FIGURE 9. Lack of bystander lysis by platelets. 

Lack of Bystander Target Cell Lysis. When labeled E or labeled EA were mixed with unlabeled EAC ~ 3b, lysis of labeled cells, if any, was not affected by the presence of unlabeled target EAC ~ 3b, as shown in Fig. 9.

Effect of Freezing and Thawing on Lytic Activity. We have found that platelets kept frozen at -70°C for >4 mo retain cytotoxic activity. To determine the effects of freezing more precisely, freshly isolated platelets were frozen and thawed three times over a 90-min period. The frozen and thawed preparation was centrifuged and the pellet was then suspended to the original volume. An assay for lactate dehydrogenase activity showed that the supernatant contained 106% of the activity of control platelets lysed with mellitin, while the pellet had only 3% activity. Thus, the platelet cytoplasmic contents were totally released by freezing and thawing. Electron microscopic examination showed that the pellet contained platelets with reduced cytoplasmic and granular contents (compare Fig. 10, A and B). These morphologic findings were supported by the retention of 34% of N-acetylgulosaminidase activity (lysosomal marker) and 21% of radiolabeled serotonin (dense granule marker) in pelleted, frozen and thawed platelets compared with untreated controls. Remaining activities were entirely recoverable in the supernatant. As shown in Fig. 11a, cytolytic activity in the particulate fraction of frozen and thawed platelets was comparable to that of intact platelets. In contrast, little activity was found in the supernate. In four of four experiments, >75% lytic activity was recovered after freezing and thawing. As with freshly isolated platelets, frozen and thawed platelets lysed EAC ~ 3b but not E.

Effect of Sonication on Lytic Activity. Platelets were disrupted by three rounds of sonication and separated into pellet and supernate by centrifugation. The pellet was resuspended to original volume. The supernate contained 100% of LDH activity, indicating that the platelets' cytoplasmic contents were totally released. As shown in Fig. 11b, the lytic activity of the sonicated, pelleted platelets was less than that of intact platelets by ~50%. Sonicated platelets specifically lysed EAC ~ 3b and not E. Lytic activity of the supernatant was negligible, in that even the undiluted supernatant caused <10% lysis of target cells. Electron microscopic examination showed that sonication disrupted platelets into small membrane vesicles with markedly diminished cytoplasmic and
granular contents (Fig. 10C). Assays for granule markers showed that the pelletable platelet fraction retained only 19% of total N-acetylglucosaminidase activity and 7% of total serotonin. In another experiment, sonicated platelets were given a hypotonic shock by exposing them to water. The lytic activity relative to intact platelets was similar to that of sonicated but not water-shocked platelets (data not shown). Likewise, the water-shocked platelets appeared similar by electron microscopy to sonicated platelets (Fig. 10D). Again, these morphologic findings were supported by granule marker assays, with 13% of total N-acetylglucosaminidase activity and 6% of total serotonin found in the pelleted fraction.

Effect of Heat or Trypsin on Lytic Activity of Platelets. Platelets were heated at 37°C for 60 min, 56°C for 60 min, or 100°C for 2 min and then lytic activities were tested. Compared with lytic activity of platelets kept at 0°C for 60 min, activity was not lost by 37°C treatment. On the other hand, heating at 56° or 100°C resulted in virtually complete loss of activity, as shown in Fig. 12a.

Figure 10. Electron micrographs of (A) intact; (B) frozen and thawed; (C) sonicated; and (D) sonicated and water-shocked platelets. (A and B) × 11,250; (C and D) × 22,500.
Figure 11. Effect of freezing and thawing or sonication on platelet lytic activity. (a) Platelets were frozen and thawed, and tested for lytic activity with E(*) or EAC ~ 3b (□). The frozen and thawed preparation was centrifuged and the resuspended pellet was assayed for lytic activity with E(○) or EAC ~ 3b (▲). The lytic activity of the supernatant did not exceed 10%. Untreated intact platelets (E) were assayed for lytic activity with EAC ~ 3b as a control. Each point represents the mean of two experiments. (b) Platelets were sonicated three times and separated by centrifugation into pellet and supernate. The pellet was resuspended to the original volume. The lytic activity of sonicated platelets with EAC ~ 3b (□) was compared with that of equivalent concentrations of intact platelets (○). As another control, the lysis of E was tested with sonicated (●) or sonicated and pelleted platelets (○). The lytic activity of undiluted as well as diluted supernatant did not exceed 10%. Each point represents the mean of two experiments.

Figure 12. Effect of heat or trypsin on platelet lytic activity. (a) Platelets were kept at 0°, 37°, or 56°C for 1 h, or at 100°C for 2 min, before assay for lytic activity. (b) Platelets were treated with trypsin at 0.01% (□) or 0.001% (▲) for 45 min and washed before assay for lytic activity. Controls incubated with buffer (○) were also tested. Each point in both panels represents the mean of two experiments.

Platelets (8 × 10⁸/ml) were incubated in buffer containing 0.001% or 0.01% trypsin at 37°C for 45 min. After incubation, the reaction medium was diluted fivefold and platelets were sedimented. Supernatants were saved and cells were washed once more in Tyrode’s-gel-EDTA before assay for lytic activity, so that the possibility of trypsin carry-over was minimized. By light microscopy, platelets appeared normal and platelet numbers were not diminished after trypsin treatment. As shown in Fig. 12b, lytic activity was markedly diminished after 0.001% or 0.01% trypsin treatment. In another experiment where trypsin was neutralized with excess soybean trypsin inhibitor (0.06%), we observed a similar loss of lytic activity. Supernatates did not contain any lytic activity for EAC ~ 3b or E.
Discussion

The participation of platelets in the destruction of antibody-coated targets such as SRBC, neoplastic cells, and schistosomal larvae has been appreciated only recently. The possible involvement of platelets in autoimmune disease has been implicated as well (33). To understand platelet-mediated cytotoxicity at a molecular level, we have developed a plasma-free experimental system which enabled us to study a number of parameters of the reaction under well-defined conditions.

In the plasma-free system, we have used washed platelets and SRBC targets sensitized with antibody or antibody and C1 through C3. Lytic efficiency in our system and in the platelet-rich plasma system described by Soper et al. (9) was similar. In most cases we found that the dose-response curve of lysis as a function of platelet concentration is entirely concave to the abscissa, indicating that a fruitful interaction between one effector and one target cell can lead to lysis. Results from numerous experiments indicate that the actual number of platelets needed to lyse a single target was in most cases about one. Our previous in vivo experiments (5–7) showed that several hundred platelets per neoplastic cell caused suppression of tumor growth. Whether or not the efficiency of in vivo destruction of neoplastic cells could be improved to the level of efficiency observed in vitro needs to be studied. In any case, considering the vast number of platelets in blood, the platelet is a major cytotoxic resource. The lag period preceding lysis suggests that multiple reaction steps ensue before lysis occurs. Temperature changes had a minor effect on lysis, i.e., Q10 of 2. Further studies are needed to determine the mechanistic significance of this observation.

We noted that target SRBC treated with subagglutinating doses of antibody alone (EA) were poorly lysed or not lysed at all. Only polyclonal rabbit IgG and monoclonal IgG2a were active, but even with these antibodies, lysis was limited. It is possible that interaction of certain isotypes of IgG, e.g., IgG2a, with appropriate Fc receptors can deliver a signal for the activation of platelet cytotoxicity, albeit an incomplete one. Interestingly, the interaction between IgG2a and its Fc receptor is believed to occur with higher affinity than those between other antibody classes and their respective Fc receptors (34). The reasons for limited activity by polyclonal IgG are more difficult to surmise, because of the heterogeneity of IgG antibodies in that preparation.

Experiments using C5-deficient plasma with and without cobra venom treatment, both in our studies and in those of Soper et al. (9), have implicated the role of C3 in triggering the lytic process. In support of this conclusion, we have found that only IgG2a, IgG2b, and IgG3, isotypes known to activate complement, were active in the reaction. However, in the study of Soper et al., non-complement-fixing isotype IgG1 was also active. Whether or not in our experiments a higher concentration of IgG1 might have triggered lysis could not be tested adequately because of target cell agglutination with more antibody. It is also of interest that we have previously shown (7) IgG1 to be active in initiating platelet-mediated tumor suppression in vivo. What factors, if any, influence activity of IgG1 in vitro and in vivo remain to be elucidated.

Our studies with IgM show that even though C3 was fixed on target cell surfaces in the presence of this antibody, lysis did not occur. Since an Fc receptor for IgM has not been identified on cytotoxic effector cells, interaction of target
cells and platelets would have taken place by means of complement receptors alone. This single type of interaction therefore is not sufficient for platelet activation. However, when equivalent amounts of C3 were fixed on target cell surfaces by IgG isotypes, e.g., IgG2b as in Table I, the two different cell-bound ligands could efficiently trigger platelet cytotoxicity. Thus, only those isotypes capable both of fixing complement and interacting with platelet Fc receptors could trigger efficient lysis.

It appears that efficient activation was achieved when the two different ligands, IgG and C3b, were adjacent to each other. Thus, IgG2b-sensitized target cells treated with complement were lysed, while target SRBC bearing equivalent amounts of cell-bound C3b generated by IgM and then sensitized with IgG2b, presumably at sites distant from the bound C3b, were not lysed. This observation in turn suggests that receptors for both ligands must be adjacent for efficient lysis to occur. Interestingly, C3b receptors and Fc receptors on human neutrophils have been reported to co-cap when either receptor was crosslinked by ligand (35).

It was reported by other investigators (11) that IgE could initiate platelet-mediated destruction of schistosomula, whereas we could not demonstrate platelet lysis of IgE-sensitized SRBC. One explanation for this apparent discrepancy could be that different targets are lysed by different mechanisms and that IgE triggers cytotoxic reactions mainly appropriate for parasitic targets.

We sought to elucidate the biochemical mechanism involved in platelet-mediated lysis of SRBC. Granular cytolysin or perforin is not likely to be involved, given the strict calcium requirement for formation of active cytolysin complexes (21–23), in contrast to the lack of a calcium requirement in platelet-mediated lysis. Our evidence suggests that proteases and toxic oxygen metabolites do not play a significant role in the lytic process.

In our initial effort to characterize the biochemical nature of the lytic system, we have found activity in the particulate fraction of frozen and thawed, sonicated, or sonicated and water-shocked platelets. The LDH release data clearly indicate that the cytoplasmic contents of platelets are not involved in the lytic reaction. The retention of lytic activity in sonicated and water-shocked platelets, as well as the absence of intact granules as shown by electron microscopy and marker assays, show that granular contents also are not involved. These results suggest that the entire recognition and lytic system is associated with the membranes of platelets. The lack of an innocent bystander effect is compatible with this notion. Isolation of these components from membranes and reconstitution of activity into liposomes would be needed to verify our proposal. The observation that heat or trypsin inactivates lytic function indicates that at least one involved component is protein. It is encouraging that the intact system can survive procedures such as freezing and thawing or sonication. Currently we are attempting to isolate the active components by various biochemical fractionation methods.

Summary

Platelets are one of several cell types capable of mediating antibody-dependent cellular cytotoxicity. We have developed a plasma-free system in which washed
mouse platelets lyse washed antibody and complement-sensitized SRBC targets in the presence of EDTA. The dose-response curve is concave to the abscissa, indicating that lysis is a one-hit reaction. Determination of the actual number of platelets required to lyse a target shows that each platelet could lyse a single target. A limited degree of lysis is observed when platelets are incubated with SRBC sensitized with monoclonal IgG2a alone, but no lysis occurs with SRBC bearing comparable amounts of other isotypes. In the presence of C1 through C3, but not C1 through C2, efficient lysis is triggered by complement-fixing monoclonal IgG2a, IgG2b, and IgG3. In contrast, IgM and non-complement-fixing IgG1 and IgE are inactive. To achieve efficient lysis, it appears that platelets require both target cell-bound antibody and C3 fragments in close proximity. It is unlikely that proteases, pore-forming proteins, or toxic oxygen metabolites are involved in platelet-mediated lysis. Freezing and thawing of platelets, sonication, or sonication followed by hypotonic shock causes severe depletion of cytoplasmic and granular contents, as shown by electron microscopy and marker assays. However, the membrane fraction of these preparations retains cytolytic activity. When platelets are treated with trypsin or heated, lytic activity is eliminated, indicating that at least one component of this system is protein. These findings, as well as the fact that platelets do not lyse unsensitized innocent bystander SRBC, suggest that the complete cytotoxic system of platelets capable of specific recognition and lysis resides in their membranes.

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