Live Cell Imaging of Outward and Inward Vesiculation Induced by the Complement C5b-9 Complex*#.

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Cells resist death induced by the complement membrane attack complex (MAC, C5b-9) by removal of the MAC from their surface by an outward and/or inward vesiculation. To gain an insight into the route of MAC removal, human C9 was tagged with Alexa Fluor 488 and traced within live cells. Tagged C9-AF488 was active in lysis of erythrocytes and K562 cells. Upon treatment of K562 cells with antibody and human serum containing C9-AF488, C9-AF488 containing MAC bound to the cells. Within 5–10 min, the cells started shedding C5b-9-loaded vesicles (0.05–1 μm) by outward vesiculation. Concomitantly, C9-AF488 entered the cells and accumulated in a perinuclear, late recycling compartment, co-localized with endocytosed transferrin-Texas Red. Similar results were obtained with fixed cells in which the MAC was labeled with antibodies directed to a C5b-9 neoepitope. Inhibition of protein kinase C reduced endocytosis of C5b-9. Kinetic analysis demonstrated that peripheral, trypsin-sensitive C5b-9 was cleared from cells at a slower rate relative to fully inserted, trypsin-resistant C5b-9. MAC formation is controlled by CD59, a ubiquitously expressed membrane complement regulator. Analysis at a cell population level showed that the amount of C5b-9-AF488 bound to K562 cells after complement activation was highly heterogeneous and inversely correlated with the CD59 level of expression. Efficient C9-AF488 vesiculation was observed in cells expressing low CD59 levels, suggesting that the protective impact of MAC elimination by vesiculation increases as the level of expression of CD59 decreases.

The complement system is a major component of the innate immune system. It efficiently protects the host from pathogenic microorganisms, is involved in immune complex regulation, and serves an important link between innate and adaptive immune responses. Complement comprises a group of more than 30 proteins; most of them participate in the cascade-like activation process, whereas others serve as control proteins or act as cellular receptors (1, 2). Initiation of the complement activation cascade may occur through the classical, alternative, or lectin pathways. The three initiation pathways lead to activation of the terminal complement pathway and to assembly of the membrane attack complexes (MACs)2 that are composed of the complement components C5b, C6, C7, C8, and C9 (also known as the C5b-9 complexes). Upon formation of C5b-7, C5b-8, and finally C5b-9 complexes, they adhere and then insert into the target cell membrane (3). The C5b-9 complexes may contain 1–18 C9 molecules attached to a C5b-8 complex. When the number of C9 molecules per C5b-8 exceeds 12, they self-polymerize and form a cylinder-shaped transmembrane structure (4). Upon its assembly, the MAC expresses neoantigens that can be detected by specific monoclonal antibodies (5). Although red blood cells require only one effective MAC to lyse, nucleated cells are killed by complement only after a combined action of several MACs (6). Although larger C5b-9 channels containing several C9 are cytolytically more potent than smaller C5b-9 channels, the channel size has only a small influence on the rate of cell death in nucleated cells (7).

To avoid accidental killing by complement, cells utilize several protective strategies, including expression of membrane complement regulatory proteins, such as CD46, CD55, and CD59 that block the activation cascades at specific stages (reviewed in Refs. 8 and 9). Second line defense is provided by intracellular resistance mechanisms that are still not characterized. Elevation of intracellular Ca2+ concentration (10, 11), protein phosphorylation by PKC and ERK (12–14), synthesis of new proteins (15), altered lipid metabolism (16), and heat shock proteins (17, 18) have all been implicated in protection. Cells protect themselves also by elimination of the MAC from the plasma membrane (19–21). C5b-8 complexes deposited on the surface of Ehrlich tumor cells were seen in endocytic vesicles and in multivesicular bodies (MVB) (21). C5b-9 complexes deposited on glomerular epithelial cells in kidneys of rats with experimental membranous nephropathy were found in clathrin-coated pits and also in MVB (20). The latter report suggested that C5b-9 complexes deposited in vivo on epithelial cells are endocytosed, packed into MVB, and then released as exocytotic vesicles into the urine. Elimination of the MAC by outward vesiculation or ectocytosis has been described in many cells (19, 20, 22–24). Shed membrane vesicles contained the

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2 The abbreviations used are: MAC, complement membrane attack complex; C5b-9, complement terminal complex composed of C5b, C6, C7, C8, and C9; C9-AF488, human complement C9 tagged with Alexa Fluor 488; C9D, C9-depleted; HI, heat-inactivated; MVB, multivesicular bodies; NHS, normal human serum; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; HBSS, Hanks’ balanced salt solution.
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MAC and elevated levels of cholesterol and diacylglycerol (25), suggesting selective membrane protein and lipid sorting during ectocytosis (23, 26).

Release of membrane vesicles from resting and activated cells is ubiquitous and has important biological implications (for a review, see Ref. 27). Vesicles shed directly from the plasma membrane by a process of outward vesiculation were named ectosomes or microparticles. In contrast, vesicles shed by a process involving endocytosis and followed by exocytosis were named exosomes. Some insight into the mechanisms involved in exosome release is beginning to emerge (28); however, the mechanism behind direct outward plasma membrane vesiculation is still elusive. Recently, the involvement of the mitochondrial hsp70 (mtHsp70/mortalin/GRP75) in shedding of C5b-9-loaded ectosomes from complement-attacked K562 cells was proposed (27). That mtHsp70 can bind directly to C9 suggested that mtHsp70 acted as a sensor identifying insertion of C5b-9 complexes into the plasma membrane and facilitating their removal by outward vesiculation (27). The purpose of this study was to design and utilize a new reagent for an in depth study of the process of outward and inward vesiculation of the C5b-9 complexes. To be able to trace C5b-9 within viable cells, purified human complement C9 was tagged with Alexa Fluor 488 and incorporated into C5b-9 complexes deposited on K562 cells. Data shown here demonstrate rapid shedding of C5b-9 complexes in small vesicles (ectosomes) directly from the cell surface. This is followed by removal of the C5b-9 via an endocytic pathway and its targeting to a late endosomal compartment, probably for degradation and/or exocytosis via MVB.

EXPERIMENTAL PROCEDURES

Sera and Reagents—Normal human serum (NHS), used as a source for complement, was prepared from healthy individuals. Heat-inactivated (HI) NHS was prepared by heating NHS for 30 min at 56 °C. Purified human C9 protein was purchased from Complement Technology, Inc. (Tyler, TX). Polyclonal antisera directed to K562 cells were prepared in rabbits and mice, and anti-human C9 antibodies were prepared in goats. Mouse monoclonal antibody directed to a neoepitope in human C5b-9 (clone aE11) was purchased from DAKO (Glostrup, Denmark). FITC-conjugated goat anti-mouse Fab, Cy3-conjugated goat anti-rabbit IgG (H + L), and peroxidase-conjugated rabbit anti-goat IgG were purchased from Jackson Immunoresearch (West Grove, PA). Rabbit anti-sheep erythrocyte antisemur (hemolysin) was purchased from Difco. Complement C9-depleted human serum (C9D-NHS), trypsin l-1-tosylamido-2-phenyl-ethyl chloromethyl ketone-treated from bovine pancreas, Heps, saponin, and HBSS were purchased from Sigma. GF190203X and PD98059 were from Calbiochem. The activity of these two inhibitors was confirmed in independent, concomitant analyses of their effects on cell lysis and on complement-induced protection (12, 13) (not shown). Transferrin Texas Red-conjugated and Alexa Fluor protein labeling kit were from Molecular Probes, Inc. (Eugene, OR).

Cell Lysis and Sublys—K562, a human erythroleukemic cell line, was cultured in RPMI 1640 supplemented with 10% (v/v) HI fetal bovine serum (Invitrogen), 2 mM glutamine, 2 mM pyruvate, and antibiotics mixture (Bio-Lab, Jerusalem, Israel). K562 cells were subcloned by limiting dilution. Briefly, K562 cells were diluted in culture medium and plated at one cell per five wells of a 96-well round bottom plate (Costar, Corning, NY). Wells containing colonies were identified under an inverted microscope (Olympus, Japan). Two K562 clones were expanded (clones A and B). Cytotoxicity assays were performed as described before (29). Briefly, cells were incubated with diluted anti-K562 antiserum (rabbit or mouse) for 30 min at 4 °C and then with complement (NHS, C9D-NHS, or HI sera; final concentration 50%) for 60 min at 37 °C. Cell lysis was determined by measuring uptake of trypan blue. In the cell imaging experiments, the cells were first treated with a sublytic dose (yielding 10–20% trypan blue positive cells) of antibody and NHS (or C9D-NHS supplemented with C9 or C9-AF488), washed, and then further incubated at 37 °C for different times.

Analysis of Secreted C9—Cells were treated with antibodies for 30 min at 4 °C and then with NHS or HI NHS (50%) for 10 min at 37 °C. Then they were extensively washed with HBSS, resuspended in HBSS, and incubated at 37 °C. At different times, the cells were removed by centrifugation at 250 × g, and the supernatant was further subjected to centrifugation at 100,000 × g. Protein concentration was determined by measuring uptake of trypan blue. In the cell imaging experiments, the cells were first treated with a sublytic dose (yielding 10–20% trypan blue positive cells) of antibody and NHS (or C9D-NHS supplemented with C9 or C9-AF488), washed, and then further incubated at 37 °C for different times.

Tagging C9 with Alexa Fluor 488—C9 was tagged with a fluorescein analogue dye, Alexa Fluor 488 (Molecular Probes). Purified human C9 (5 μg) was treated with the dye reagent according to the manufacturer’s instructions. The reagent mixture was stirred at room temperature for 60 min, and the unbound dye was removed by using a spin column. The level of fluorescence of tagged C9-AF488 was analyzed in a microplate reader (Spectrafluor plus, Tecan, Austria). Concentration of C9-AF488, relative to serially diluted C9 stock, was determined by SDS-PAGE followed by silver staining of the gel and densitometric analysis of stained protein bands (supplemental Fig. S1). Concentration of C9-AF488 was determined to be 21.7 μg/mL. The molar ratio of AF488 to C9 in C9-AF488 was calculated after measuring the fluorescence units of samples of C9-AF488 and of serially diluted AF488 reagent of known concentrations in a microplate reader (supplemental Table 1 and Fig. S2). Tagged C9-AF488 contained 1.67 mol of fluorophore/mol of C9. Hemolytic Activity of C9-AF488—Antibody-sensitized sheep erythrocytes were prepared with rabbit hemolysin (Difco) as described before (30). The antibody-coated sheep erythrocytes (1.5 × 10^7) in 0.1 ml of gelatin/veronal-buffered saline containing 1 mM MgCl2 and 0.1 mM CaCl2 (GVB) were incubated with C9-depleted human serum (diluted 1:2,000) and increasing amounts of C9 or C9-AF488 for 30 min at 37 °C. Cold GVB (1
ml) was added to stop lysis, and the cells were removed by centrifugation. The percentage of hemolysis was calculated from the light absorption of the cell supernatants at 412 nm, relative to cells completely lysed by water. C9-AF488 was found to be hemolytically active (Fig. S3).

**Flow Cytometry Analysis of C5b-9 Deposition—**K562 Cells were incubated with a sublytic dose of diluted anti-K562 antiserum for 30 min at 4 °C and then with complement (NHS or HI NHS, 50%) for 10 min at 37 °C. Next, the cells were washed into HBSS and incubated at 37 °C for different times and finally fixed with 1% paraformaldehyde for 15 min at 4 °C. For permeabilization, cells were washed with phosphate-buffered saline supplemented with 1 mg/ml saponin and 5 mg/ml bovine serum albumin and then treated with phosphate-buffered saline containing 1 mg/ml saponin, 5 mg/ml bovine serum albumin, and 10% fetal calf serum for 20 min at 4 °C. Permeabilized cells (for measurement of total MAC) and nonpermeabilized cells (for measurement of surface MAC) were labeled with aE11 monoclonal antibody and a second FITC-labeled antibody and analyzed in a FACSort (BD Biosciences). The fluorescence data (7,000 cells) were analyzed by using WinMDI 2.8, and mean fluorescence intensity (G-mean) values were determined. Control cells were treated with second antibody alone.

**C5b-9 Imaging by Confocal Microscopy—**Cells were incubated with sublytic antibody and NHS, HI-NHS, or C9-depleted serum supplemented with C9-AF488 for 10 min at 37 °C. Next, they were washed into HBSS and incubated at 37 °C for different times. In some experiments, the cells were fixed with 1% paraformaldehyde and either permeabilized with saponin or not. The cells were immunolabeled with aE11 or with anti-CD59 and a second FITC-labeled or Cy3-labeled antibody, respectively. Cells were analyzed under a Zeiss laser confocal microscope C-LSM 410 (Oberkochen, Germany). Images and merged images were obtained with the LSM software (Carl Zeiss, GmbH, Germany). For time lapse imaging of viable cells, the cells were treated with sublytic antibody and then with human C9-depleted serum supplemented with C9-Alexa 488 for 0 or 5 min at 37 °C. Then the cells were washed or not with HBSS and placed on a 22-mm coverslip (Assistant, Sondheim, Germany). Time lapse image capturing was started a few seconds after warming of the specimen to 37 °C, in intervals of 10 or 20 s over 10 or 20 min. Images were processed further for display by using Image J (National Institutes of Health, Bethesda, MD). Minimal laser intensities were used for image collection in order to minimize risks of photobleaching and phototoxicity.

**Analysis of Transferrin Endocytosis—**Cells were treated with antibody and complement, as described above. Then they were incubated with Texas Red conjugated transferrin (Molecular Probes) in binding buffer (RPMI 1640, 20 mM Hepes, pH 7.4, and 1 mg/ml bovine serum albumin) for 30 min at 4 °C and further incubated at 37 °C for different times. After washing and fixation, the cells were analyzed by confocal microscopy, as described above.

**Statistical Analysis—**Student's two-sided paired *t* tests were used to determine the statistical significance of differences between various data sets. Results are expressed as arithmetic mean ± S.D. Statistical significance was assumed when *p* < 0.05.

**RESULTS**

**Formation of Active C9-AF488—**Human C9 protein was tagged with Alexa Fluor 488 as described under "Experimental Procedures." This C9-AF488 was shown to be hemolytically active when tested with antibody-coated sheep red blood cells (Fig. S3). To test the capacity of C9-AF488 to induce lysis of K562 cells, the cells were treated with anti-K562 antibodies, followed by C9D-NHS or HI-C9D-NHS supplemented with C9-AF488 (to physiological concentration of C9) for 60 min at 37 °C. Whereas C9D-NHS failed to lyse K562 cells (not shown), in the presence of C9-AF488, C9D-NHS induced K562 cell lysis (Fig. 1). The antibody titration curve observed was similar to that we routinely obtain with antibody and NHS. K562 cells were not lysed by HI-C9D-NHS supplemented with C9-AF488 (Fig. 1).

**Emission of C9-loaded Vesicles—**Several cell types have been shown to eliminate the MAC by ectocytosis (outward vesiculation) (19, 22, 24, 26). Here, C9-AF488 was used to study the kinetics of formation and trafficking of C5b-9-bearing ectosomes. K562 cells were treated with anti-K562 antibodies and then with C9D-NHS supplemented with C9-AF488 for 10 min at 37 °C. As shown before, this treatment yields maximal deposition of C5b-9 on the cells (31). Next, the cells were incubated in HBSS at 37 °C and analyzed under a confocal microscope. Fluorescence and bright field images showed rapid formation of large vesicles (~1 μm in diameter) labeled with C9-AF488, at the cell surface (Fig. 2A). Time lapse photography of viable C5b-C9-AF488-labeled cells clearly demonstrated rapid formation and emission of numerous C9-loaded vesicles. Fig. 2B shows formation and release of such an ectosome that lasts about 80 s from budding to pinching off (see also Movie S1). Few cells appeared also to release C9-AF488-loaded vesicles following accumulation of C9-AF488 below the plasma membrane (Fig. 2C; see also Movie S2). Since these cells still did not have clusters of C9-AF488-tagged C5b-9 in MVB (see below), this might represent a novel type of early endocytosis leading to
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A  C9-AF488  Phase contrast

B
9'00"  9'20"  9'40"  10'00"  10'20"

10'40"  11'00"  11'20"  11'40"  12'00"

C
8'20"  9'00"

8'40"  9'20"

D

Sup
Pellet
TX-100: - +
direct vesicle release. Analysis of the sedimentation capacity of the C9 shed from the cells demonstrated that at least 51% of it could be spun down at 100,000 \( \times \) g, probably attached to membrane vesicles, and became soluble after treatment with Triton X-100 (Fig. 2D).

**Endocytosis of the C5b-9 Complex**—Inward vesiculation of the MAC was examined in viable K562 cells by using C9-AF488 and in fixed/permeabilized cells labeled with the aE11 antibody detecting a neo-C5b-9 antigenic epitope. C9-AF488 was seen inside the K562 cells as early as 5 min after maximal binding and cell washing (altogether 15 min at 37 °C) (Fig. 3). Time lapse photography demonstrated trafficking of C9-AF488-labeled intracellular vesicles from the cell periphery (early endosomal compartment) to a perinuclear compartment, probably the late endosomal region containing the MVB (Fig. 3; also see Movie S3). The MVB compartment reached its maximal size at 15–30 min after cell washing. Similar results were observed with cells treated with antibody and NHS, fixed, permeabilized, and labeled with aE11 that detects the native form of C5b-9 (not shown). This indicated that the rate and mode of clearance of C5b-9 complexes formed with C9-AF488 or native C9 were similar. Intracellular trafficking of C9-AF488 was compared with that of transferrin. C9-AF488 and transferrin-Texas Red entered K562 cells at about the same speed, and both reached the late recycling compartment after 30–40 min (Fig. 4A). Furthermore, the fact that the transferrin and the C9 fluorescence merged at the perinuclear region (Fig. 4, B [C9-AF488] and C [aE11 mAb]) indicated that the cells transported the intracellular C5b-9 complexes to the late endosomal MVB-containing region. Even after 40 min, part of the C5b-9 complexes remained on or next to the cell surface (Fig. 4). This finding will be further addressed below. To confirm that cell entry of C9-AF488 required active C9 deposition and C5b-9 formation, we treated cells with heat-inactivated C9-depleted serum or C8-deficient human serum supplemented with C9-AF488 and found no intracellular C9-AF488 (data not shown).

**Kinetics of Removal of Deposited C5b-9**—Activation of the terminal pathway of complement leads to insertion of the C5b-9 complex into the plasma membrane. However, some of the complexes fail to penetrate the plasma membrane but remain attached to the cell surface. Unlike the inserted complexes, surface-attached complexes are sensitive to trypsin treatment (32). Cells were treated with sublytic antibody and complement, and the amount of surface-bound C5b-9 complexes was assessed by flow cytometry in viable cells labeled with the aE11 monoclonal antibody. To distinguish between transmembrane and surface-attached C5b-9, we treated C5b-9-bearing cells with trypsin before aE11 staining (supplemental Fig. S4). At time 0, after 10-min complement treatment at 37 °C, ~55% of the total deposited C5b-9 was found to be trypsin-sensitive (i.e. exogenously attached to the cell surface) (Fig. 5A). Similar analyses performed after 20, 40, and 60 min showed an increase in the relative amount of the externally attached C5b-9 to 60, 77, and >90%, respectively (Fig. 5A). The rates of elimination of transmembrane and externally attached C5b-9 were calculated based on the mean fluorescence intensities of the aE11/C5b-9 histograms. As shown in Fig. 5B, fully inserted, transmembrane C5b-9 (trypsin-resistant) was eliminated from the cell surface at a faster pace \( (t_{1/2} = 22\text{ min}) \) compared with externally bound (trypsin-sensitive) C5b-9 \( (t_{1/2} = 33\text{ min} \text{ between} \ 0 \text{ and} \ 20 \text{ min and} \ 106\text{ min between} \ 20 \text{ and} \ 60 \text{ min}) \). Elimination half-time of total surface C5b-9 (sensitive and resistant to trypsin) was 38 min.

**Endocytosis of C5b-9 Is a PKC-dependent, ERK-independent Process**—PKC and ERK are known to play a role in protection of K562 cells from complement-mediated damage (12–14) and in outward vesiculation of mortalin (mitochondrial hsp70) (27). Possible involvement of PKC and/or ERK in endocytosis of C5b-9 was next examined. K562 cells were pretreated with 10 \( \mu \text{M} \) GF109203X (a specific PKC inhibitor) or PD98059 (a specific ERK inhibitor) or with Me2SO as control and were then treated with sublytic anti-K562 antibodies and with NHS. Next, the cells were labeled with aE11 monoclonal antibody and analyzed by flow cytometry as described. Total amount of C5b-9, inside the cells and on the cell surface, was determined by staining of fixed/permeabilized cells with aE11. The amount of surface-bound C5b-9 complexes was determined with viable cells labeled with the aE11 monoclonal antibody. The amount of intracellular C5b-9 was derived by subtraction of the mean fluorescence intensities of the surface bound aE11/C5b-9 from the total bound C5b-9. Preliminary analysis demonstrated that over a period of 60 min at 37 °C, the amount of surface-bound C5b-9 gradually decreased (Fig. S5A), and the amount of intracellular C5b-9 increased (Fig. S5B). However, most of the internalization occurred within the first 20 min. Therefore, the effect of the kinase inhibitors on endocytosis was tested at 20 min after C5b-9 deposition and cell washing. As shown in Fig. 6A, GF109203X had no effect on the total amount of C5b-9 deposited on the cells and on its distribution between the intracellular and extracellular compartments. However, 20 min later, the amount of intracellular C5b-9 was significantly lower in GF109203X-treated cells as compared with control cells (Fig. 6B). Accordingly, more C5b-9 was seen on the surface of GF109203X-treated cells. In contrast, pretreatment with
PD98059 led to a small increase in the total amount of deposited C5b-9 (Fig. 6C) but had no effect on endocytosis of the C5b-9 complexes (Fig. 6D).

Extent of C5b-9 Deposition on K562 Cells Is Inversely Related to the Amount of CD59—Analyses of C5b-9 deposition on K562 cells by using C9-AF488 or aE11 showed a heterogeneous distribution, with cells having high levels of C5b-9 and cells having almost no C5b-9 (e.g. Fig. 4A). Subcloning of K562 cells and analysis of C5b-9 deposition on two of the subclones yielded similar results. C5b-C9-AF488 deposition on the K562 cloned cells was still very heterogeneous (Fig. S6). The possibility that CD59, a known inhibitor of C5b-9 formation (33), is enforcing this heterogeneous deposition of C5b-9 was examined. K562 cells were treated with anti-K562 antibodies and then with C9D-NHS + C9-AF488 (10 min at 37 °C) were washed and analyzed under a confocal microscope. A series of pictures of a single cell taken between 5 and 15 min, every 30 s, is shown. This cell represents hundreds of similar cells observed in five independent experiments. An arrow points at C9-AF488 accumulating in a perinuclear compartment. An arrowhead points at a small vesicle (~50 nm in diameter) pinching off from the cell surface.
CD59 and a second Cy3-labeled antibody. The cells were examined by confocal microscopy, and the distribution of C9-AF488 and CD59/Cy3 was examined. As shown in Fig. 7, A–C, cells expressing higher levels of CD59 expressed lower levels of C5b-9. This inverse correlation was found to be significant (Fig. 7D, p < 0.05). A similar analysis was performed to correlate the amount of bound anti-K562 antibodies with the level of deposition of C5b-9. There was no correlation between C5b-9 deposition and anti-K562 binding (Fig. 7E). A possible inhibitory effect of bound C5b-9 on binding of anti-CD59 antibody to CD59 was ruled out (Fig. S7). Hence, it appears that a heterogeneous expression of CD59 in the K562 population is affecting the level of C5b-9 deposition on these cells.

DISCUSSION

Pore-forming proteins like complement C9 (34) and bacterial toxins (35) form oligomers, sink into the plasma membrane, and activate scores of activities by mechanisms that are still not well understood. At sublytic doses, they signal cells to survival, activation, transformation, differentiation, or even division (36–39). At high doses, these proteins are toxic and lethal, possibly by dysregulating intracellular calcium and causing damage to cellular organelles (36, 40, 41). Cells have developed means for handling such toxic pore formers and limiting their number to sublytic amounts. This study aimed at gaining a deeper insight into these mechanisms. Our results present a novel method for tracking the traffic of the pore former complement C9, tagged with Alexa Fluor 488, in living cells by laser confocal microscopy. It is conceivable that a similar technique may be used to study the intracellular trafficking of other pore-forming proteins. The advantage of this technique is 2-fold; it permits a real time investigation in living cells, and it does not require a second labeling step with a fluorophore-tagged reagent. The results presented here demonstrate that soluble C9-AF488 is deposited within 10 min on the surface of K562 cells, probably as a subcomponent of the C5b-9 complex, and that this is pursued by an immediate clearance activity. Our live cell image
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FIGURE 6. Endocytosis of the C5b-9 depends on PKC but not on ERK. K562 cells were pretreated with 10 μM GF109203X (GF), 10 μM PD98059 (PD), or 0.1% Me2SO (DMSO) (solvent control) for 30 min at 37 °C. Next, they were treated with antibody and with NHS (10 min at 37 °C), washed, and incubated again at 37 °C for 20 min. Permeabilized cells (Total C5b-9) and nonpermeabilized cells (Surface C5b-9) were labeled with aF11/FITC second antibody and analyzed by flow cytometry. See Fig. S5 for more details. The difference between the mean fluorescence intensities of total C5b-9 and surface C5b-9 was defined as the apparent mean fluorescence intensity of intracellular C5b-9 (IC). Mean results ± S.E. of four experiments are shown. A, GF- and Me2SO-treated cells at time 0. B, PD98059- and Me2SO-treated cells at time 0. C, PD98059- and Me2SO-treated cells at time 0. D, PD98059- and Me2SO-treated cells after 20 min.

analyses show rapid (within 5–10 min) outward and inward vesiculation of C9-AF488, demonstrating “shooting” of C9-AF488-loaded vesicles (0.05–1-μm diameter) from the cell surface as well as exocytosis of C9-AF488-loaded vesicles that have undergone a rapid recycling through the cell cortex. Ten min later, C9-AF488 begins to accumulate in the early endosomal compartment and later (>20 min) in a perinuclear central compartment. Co-assembly of C9-AF488 with transferrin-Texas Red in the perinuclear compartment suggests that the C5b-9 complex is being transported to late recycling endosomes. There, they may either be packed within the multivesicular bodies and transported for exocytosis or be subjected to proteolytic degradation. These observations have been reproduced by indirect immunofluorescence with fixed and permeabilized cells labeled with an antibody directed to a neoepitope on the C5b-9 complex.

Kinetic analysis of the rate of elimination of C5b-9 from the K562 cell surface indicates an average half-life of 38 min (Fig. 5). However, this figure represents averaged decay of two distinct C5b-9 populations; one is trypsin-resistant and decays with a half-life of 22 min, and a second one is trypsin-sensitive and decays first with a half-life of 33 min and later 106 min. Resistance to trypsin is conferred on C5b-9 upon its complete insertion into the plasma membrane (32). Hence, our data suggest that cells remove the inserted (and potentially toxic) C5b-9 much (~5 times) faster than peripheral, nontoxic C5b-9. It is conceivable that the sorting machinery that assembles C5b-9 for vesiculation identifies the inserted C5b-9 and less so the peripheral C5b-9. Our earlier results have shown that the mitochondrial hsp70 (mtHsp70/mortalin/grp75) is capable of binding to complement C8 and C9 and is released from K562 cells together with C5b-9 (27). The possibility that mtHsp70 is the chaperone that identifies the inserted C5b-9 and targets it for vesiculation is being further examined. Our finding that the addition of anti-mtHsp70 antibodies can reduce outward vesiculation of C5b-9 and mtHsp70 (27) supports this hypothesis.

Semi-quantitative analyses (Figs. 6 and S5) have demonstrated that although a large fraction of the deposited C5b-9 is removed from K562 cell surface by inward vesiculation, a relatively smaller fraction of the C5b-9 is removed by outward vesiculation. This is based on the observation that the total amount of deposited C5b-9 (surface plus intracellular) begins to significantly decrease only at the 60 min incubation time point. Thus, the blocking effect of the anti-mtHsp70 antibodies may be interpreted as suggesting that these antibodies, in the extracellular milieu, bound mtHsp70 and prevented somehow both outward and inward vesiculation. Alternatively, it is possible that outward vesiculation is functionally more protective from cell death than inward vesiculation. Neutrophils also eliminate C5b-9 by outward and less so by inward vesiculation (19). Inward vesiculation of the C5b-9 has been demonstrated in glomerular epithelial cells and Ehrlich tumor cells (19–21), whereas outward vesiculation was reported in oligodendrocytes, platelets, and the tumor cell lines Ehrlich and U937 (19, 21–24). As shown here, release of both ectosomes and exosomes are rapid processes, and future studies will clarify their relative impact on cell resistance to MAC-mediated cell death.

PKC and ERK are known to play a role in protection of K562 cells from complement-mediated damage (12–14). C5b-9 elimination was suggested to be dependent on PKC activation (14). In agreement with this suggestion, our results demonstrated that although inhibition of PKC had no effect on the level of C5b-9 deposition (Fig. 6A), it significantly enhanced the amount of C5b-9 retained on the cell surface after 20 min of decay (Fig. 6B). This indicates that PKC is involved in the internalization process of C5b-9. Cell triggering by complement (13) and by many other stimuli (42, 43) is known to induce translocation of PKC to the plasma membrane. Recent studies have identified a novel translocation of PKC to a juxtanuclear compartment, the pericentron, which is distinct from the Golgi complex but epicentered on the centrosome. Sustained activation of PKC also results in sequestration of plasma membrane lipids and proteins to the same compartment (44). Possibly, PKC contributes to cell protection from complement by facili-
C5b-9 deposition is inversely correlated with the level of CD59 expression. A–C, K562 cells were treated with a sublytic dose of mouse anti-K562 antibodies and then with C9D-NHS supplemented with C9-AF488 for 10 min at 37 °C. Next, the cells were washed, fixed, permeabilized, and labeled with anti-CD59 and a second Cy3-labeled antibody. The cells were analyzed under a confocal microscope. The arrows point at two cells that had inverse cell images. A significant inverse correlation is shown between the levels of C9-AF488 and CD59 (fluorescence units; F.U.) was similarly quantified in 200 randomly chosen cell images. B, C, and D, levels of C9-AF488 and CD59 (F.U.) were quantified with Image J in 100 randomly chosen cell images. A significant inverse correlation is shown between the levels of C9-AF488 and CD59 (R² = 0.9527; p < 0.05). E, the level of bound mouse anti-K562 antibody (mAK562) was similarly quantified in 200 randomly chosen cell images by using a second Cy3-labeled anti-IgG antibody. No correlation was found between the level of C5b-C9-AF488 deposition and amount of antibody binding (R² = 0.0005).

FIGURE 7. C5b-9 deposition is inversely correlated with the level of CD59 expression. A–C, K562 cells were treated with a sublytic dose of mouse anti-K562 antibodies and then with C9D-NHS supplemented with C9-AF488 for 10 min at 37 °C. Next, the cells were washed, fixed, permeabilized, and labeled with anti-CD59 and a second Cy3-labeled antibody. The cells were analyzed under a confocal microscope. The arrows point at two cells that had inverse cell images. A significant inverse correlation is shown between the levels of C9-AF488 and CD59 (fluorescence units; F.U.) was similarly quantified in 200 randomly chosen cell images. B, C, and D, levels of C9-AF488 and CD59 (F.U.) were quantified with Image J in 100 randomly chosen cell images. A significant inverse correlation is shown between the levels of C9-AF488 and CD59 (R² = 0.9527; p < 0.05). E, the level of bound mouse anti-K562 antibody (mAK562) was similarly quantified in 200 randomly chosen cell images by using a second Cy3-labeled anti-IgG antibody. No correlation was found between the level of C5b-C9-AF488 deposition and amount of antibody binding (R² = 0.0005).

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