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The cytolytic C5b-9 complement complex: Feedback inhibition of complement activation

(complement regulation/C3 convertase formation)

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ABSTRACT **We describe a regulatory function of the terminal cytolytic C5b-9 complex [C5b-9(m)] of human complement. Purified C5b-9(m) complexes isolated from target membranes, whether in solution or bound to liposomes, inhibited lysis of sensitized sheep erythrocytes by whole human serum in a dose-dependent manner. C9 was not required for the inhibitory function since C5b-7 and C5b-8 complexes isolated from membranes were also effective. No effect was found with the cytolytically inactive, fluid-phase SC5b-9 complex. However, tryptic modification of SC5b-9 conferred an inhibitory capacity to the complex, due probably to partial removal of the S protein. Experiments using purified components demonstrated that C5b-9(m) exerts a regulatory effect on the formation of the classical- and alternative-pathway C3 convertases and on the utilization of C5 by cell-bound C5 convertases. C5b-9(m) complexes were unable to inhibit the lysis of cells bearing C5b-7(m) by C8 and C9. Addition of C5b-9(m) to whole human serum abolished its bactericidal effect on the serum-sensitive *Escherichia coli* K-12 strain W 3110 and suppressed its hemolytic function on antibody-sensitized, autologous erythrocytes. Feedback inhibition by C5b-9(m) represents a biologically relevant mechanism through which complement may autoregulate its effector functions.**

Activation of the entire complement sequence by the classical or the alternative pathway results in the formation either of a membrane-bound C5b-9(m) complex or of an inactive SC5b-9 complex in the fluid phase (1–3). Cell-bound C5b-9(m) generates functional transmembrane pores (1–6), a process that may be followed by lysis of target cells and/or by secondary cellular responses such as neosynthesis of arachidonic acid metabolism derivatives (7–9). To date, these effects on attacked membranes and target cells are the sole recognized functions of C5b-9(m) complexes.

Each step during complement activation is controlled by regulatory proteins that are normally present in plasma and/or on cell surfaces (10–15). In no case has a product of complement activation itself been found to down-regulate one or several steps in the complement sequence. In this communication, we report a regulatory function of the membrane-bound C5b-9(m) complex. We have found that this complex, but not its fluid-phase cytolytically inactive SC5b-9 counterpart, is capable of suppressing formation of classical- and alternative-pathway C3 convertases and can inhibit the function of preformed cell-bound C5 convertase. In whole human serum, C5b-9(m) complexes inhibit the lysis of heterologous and autologous antibody-coated erythrocytes and abrogate killing of a serum-sensitive strain of *Escherichia coli*. Feedback inhibition of complement activation by C5b-9(m) represents a hitherto unrecognized function of the terminal complex.

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MATERIALS AND METHODS

Isolation of Terminal Complement Complexes and Liposome Preparation. Terminal C5b-7, C5b-8, and C5b-9 complement complexes were generated on rabbit erythrocytes by incubation of cells with pooled, whole human serum or with sera immunologically depleted of either C8 or C9 (16–18). Membranes were solubilized with 125 mM deoxycholate, and the respective terminal complexes were isolated through sucrose density centrifugation in detergent (deoxycholate) (16–18). Detergent was removed by a passage of the protein over Sephadex G-25 (PD 10 columns from Pharmacia) equilibrated with Veronal-buffered saline (VBS); this step was performed immediately prior to the lysis inhibition experiments.

Purified C5b-9(m) complexes were reincorporated into lipid membranes as detailed earlier (5). Protein recovered from the sucrose density gradients was added to phosphatidylcholine (Sigma, type V-E) dissolved in 125 mM deoxycholate at protein:lipid ratios of 1:20 (wt/wt). After 3 days of dialysis at 22°C against 500 vol of detergent-free buffer (20 mM Tris·HCl/50 mM NaCl, pH 8), the liposomes were separated from unbound protein by flotation through sucrose solutions as described (5). The top fraction containing liposomes with incorporated C5b-9(m) was dialyzed against saline and protein was determined by quantitative amino acid analysis. Control liposomes were prepared in the absence of C5b-9(m).

Fluid-phase SC5b-9 complexes were isolated from inulin-activated serum as described (19). Purified SC5b-9 (1.3 mg/ml solution in VBS) was trypsinized (final enzyme concentration, 10 μg/ml) for 16 hr at 22°C, after which soybean trypsin inhibitor (Merck) was added at a final concentration of 20 μg/ml (20).

Inhibition of Lysis of Sensitized Sheep Erythrocytes (E^s). One volume of E^s sensitized with rabbit anti-E^s stroma antibodies (E^sA) (1.5×10^8 cells per ml) in VBS containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{2+}) was incubated with 1 vol of 2% whole normal human serum (NHS) in the presence of terminal complement complexes at 37°C, and hemolysis was measured after 60 min. In another set of experiments, unlysed cells were washed twice in VBS/10 mM EDTA and then incubated with 10% NHS/10 mM EDTA.

Inhibition of Formation of Classical-Pathway Cell-Bound C3 Convertase Sites. VBS and VBS^{2+} containing 0.1%

Abbreviations: C5b-7(m), C5b-8(m), and C5b-9(m), complement complexes of C5b-C7, C5b-C8, and C5b-9 formed on membranes; E^s, sheep erythrocyte(s): NHS, normal human serum; E^sA, antibody-coated sheep erythrocyte(s).

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gelatin (GVB and GVB^{2+}, respectively), half-isotonic GVB and GVB^{2+} with 2.5% dextrose (DGVB and $DGVB^{2+}$, respectively), and GVB containing 0.04 M EDTA (GVB/EDTA) were prepared as described (21).

E^s bearing human C4b and functionally purified guinea pig C1 (E^sAC14b) (1×10^7) were allowed to react with 0.02 μg of human C2 (Cordis, Miami, FL) in 0.20 ml of DGVB containing 0.15 mM Ca^{2+} and 0.05 mM Ni^{2+} ($DGVB/Ni^{2+}$) in the presence of various amounts of C5b-9(m) for 7 min at 30°C. Guinea pig serum diluted 1:50 in GVB/EDTA (0.3 ml) was then added to the reaction mixture with or without prior washes of the cells in GVB/EDTA, and incubation was continued for 60 min at 37°C. The number of C3 hemolytic sites per cell in the samples incubated with C5b-9(m) was calculated and expressed as percentage of C3 hemolytic sites in the sample in which convertase sites were formed in the absence of C5b-9(m).

Inhibition of Formation of Alternative-Pathway C3 Convertase Sites. Human C3 (22), B (23), and D (24) were purified to homogeneity from normal human plasma. E^sA bearing human C4b and C3b (E^sAC43b) (25) were prepared as indicated. E^s bearing C3b were prepared (26) by incubating E^s (1×10^9) with 1.0 mg of C3, 0.20 mg of B in 1.0 ml of GVB^{2+} containing excess D, and 5 mM additional Mg^{2+} for 45 min at 30°C. The cells were washed in GVB^{2+}, incubated with 80 μg of B in 1.0 ml of $DGVB^{2+}$ containing 5 mM additional Mg^{2+} for 30 min at 30°C, washed in ice-cold $DGVB^{2+}$, and further incubated in 1.0 ml of $DGVB^{2+}$ containing 450 μg of C3 for 45 min at 30°C. The cells were washed in $DGVB^{2+}$ and the second step of C3b deposition was repeated once.

Concentrations of E^sC3b or E^sAC43b (1×10^8 per ml), B, and D in $DGVB^{2+}/Ni^{2+}$ were then adjusted to generate ≈1.5 hemolytic sites per cell in the absence of C5b-9(m); 0.1 ml of the reaction mixture was added to 0.1 ml of buffer containing various concentrations of C5b-9(m). After incubation for 30 min at 30°C, convertase sites were revealed by addition of 0.3 ml of a 1:20 dilution of rat serum in GVB/EDTA and further incubation for 60 min at 37°C. Inhibition of C3 convertase formation was calculated as described above.

Assessment of C3 Cleavage by Preformed Classical-Pathway C3 Convertase Sites. Cells (2.5×10^8) bearing C4b,2a sites were prepared in $DGVB^{2+}/Ni^{2+}$ (27). After centrifugation and washing, the cells were resuspended in 0.6 ml of a 1:20 dilution of human C2-deficient serum in $DGVB^{2+}/Ni^{2+}$ in the presence or absence of 120 nM C5b-9(m), and incubation was carried out at 37°C. At various time points, the amount of C3 cleaved was assessed by measuring generation of C3adesArg in the fluid phase by using the radioimmunoassay from Upjohn (28). C2-deficient serum was used to avoid generation of new classical-pathway convertase sites on $E^sAC4b,2a$ during the course of the experiment.

Assessment of C5 Utilization by Preformed Cell-Bound C5 Convertase Sites. Stabilized classical pathway C5 convertase sites $C4b,2a(C3b)_n$ were generated by forming $E^sAC4b,2a$ cells in the presence of 0.05 mM Ni^{2+} as described above and then incubating the C3 convertase-bearing cells (1×10^8) with purified human C3 (14 μg) for 15 min at 30°C. The cells were centrifuged, washed, and resuspended in DGVB/EDTA. Addition of C5b-9(m) to these cells for 20 min, followed by washing and incubation with a source of C5–C9, revealed <15% decay of preformed C5 convertase sites. To investigate inhibition of C5 utilization by the C5 convertase in the presence of C5b-9(m), stabilized C5 convertase-bearing cells [$E^sAC4b,2a(3b)_n$] (1×10^7) were incubated with 0.2 μg of purified human C5 (29) in the presence of various concentrations of C5b-9(m) in 0.2 ml of GVB/EDTA for 4 min at 30°C. Three hundred microliters of a 1:10 dilution of rat serum that had been depleted of C3 and C5 by using 1 M KSCN and 0.015 M hydrazine as the source of C6–C9 (30) was then added and the reaction mixture was incubated for 60 min at 37°C.

Bactericidal Assays. The serum-sensitive *E. coli* K-12 strain W 3110 (28) was suspended at 1×10^8 colony-forming units/ml in VBS and incubated with 2% whole NHS in the presence or absence of C5b-9(m) for 20 min at 37°C. Samples were appropriately diluted and plated out on agar cultures. Bactericidal activity was assessed by colony counting (31).

Lysis of Autologous Cells. Washed human erythrocytes (10^8 cells per ml in VBS^{2+}) were treated with polyspecific rabbit antibodies against human erythrocyte membrane proteins (code A 104 from Dakopatts, Copenhagen) at a final antibody dilution of 1:300 for 15 min at 22°C. Thereafter, the cells were washed twice and resuspended to 10^8 cells per ml, and hemolysis tests were conducted as described for E^sA above with autologous serum at a final concentration of 4%.

RESULTS

Inhibition of Lysis of E^sA by Terminal Complement Complexes. When sensitized E^sA were incubated with whole NHS under conditions that would cause 85–90% hemolysis, the addition of purified C5b-9(m) complexes in nanomolar concentrations to the reaction mixture resulted in a dose-dependent reduction of hemolysis (Fig. 1). Fifty-percent inhibition of E^sA lysis was achieved with 10–12 nM C5b-9(m). The effect was also found with purified C5b-7(m) and C5b-8(m), indicating that C9 was not required for the observed inhibitory function (Fig. 1). On a molar basis, the higher concentration of C5b-7(m) required for inhibition may be due to the strong aggregation tendency of this complex in detergent-free solution. If unlysed cells were washed and then treated with EDTA/NHS, <25% lysis ensued, indicating that the major inhibitory step occurred prior to the stage of C5b-7 formation.

The addition of C5b-9(m) complexes to whole serum results in binding of the S protein to the apolar surfaces of C5b-9(m) to generate water-soluble SC5b-9(m) complexes, which differ from naturally formed SC5b-9 complexes that occur during fluid-phase complement activation (2, 16). The inhibitory function of C5b-9(m) on E^sA lysis was independent of S-protein binding to the complex. Thus, inhibition was also observed when C5b-9(m) was first incorporated into liposomal membranes and the membrane-bound complexes were added to serum (Fig. 2). Control analyses confirmed that S protein did not significantly bind to such liposome-incorporated C5b-9(m) complexes. These results indicated that S protein is not itself involved in the inhibition process and that C5b-9(m) will exert a negative feedback control over the cytolytic pathway in its membrane-bound form at the site of its generation.

In contrast to membrane-derived C5b-9(m), freshly prepared SC5b-9 complexes generated by inulin activation of whole human serum failed to inhibit E^sA lysis (Fig. 2*B*). Removal of S protein by trypsination endowed this complex with inhibitory activity that was, however, weaker than that observed at equimolar concentrations of C5b-9(m) (Fig. 2*B*). These findings suggest that binding of S protein during generation of fluid-phase SC5b-9 masks the site(s) within the C5b-7 moiety of the complex that is involved in its regulatory function, and partial removal of S protein partially unmasks the inhibitory capacity of the complex.

C5b-9(m) Inhibits Formation of Classical- and Alternative-Pathway C3 Convertases. The following experiments demonstrated that a first site of inhibitory C5b-9(m) action is at the stage of C3 convertase formation. Classical-pathway C3 convertase sites were generated on E^sAC14b by incubation with human C2 in the presence of 0.05 mM Ni^{2+}. The cells were washed and convertase sites were revealed by addition of 2% EDTA/guinea pig serum as a source of C3–C9.

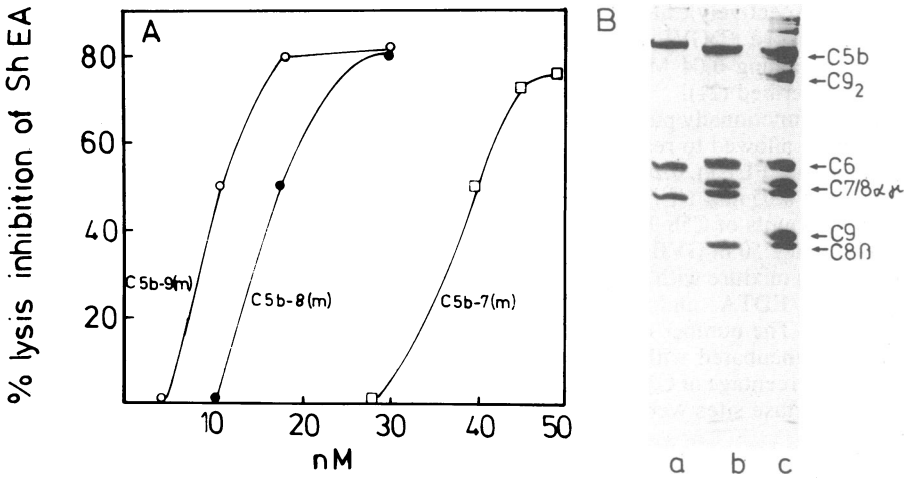

FIG. 1. (*A*) Inhibition of sensitized E^{s}A (Sh EA) lysis by whole human serum in the presence of nanomolar concentrations of purified C5b-7(m), C5b-8(m), and C5b-9(m). One volume of E^{s}A (1.6×10^8 cells per ml) was incubated with 1 vol of 2% whole human serum in the presence of terminal complement complexes and lysis was measured after 60 min at 37°C. (*B*) NaDodSO$_4$/PAGE patterns of the purified terminal complexes used in the experiment. Lanes: a, c5b-7; b, c5b-8(m); c, C5b-9(m). Subunit designations are as in ref. 2.

Alternative-pathway C3 convertase sites were formed by incubating E^{s}C3b or E^{s}AC4b3b with purified B and D in the presence or absence of 0.05 mM Ni^{2+}. The cells were washed and convertase sites were revealed by addition of 5% EDTA/rat serum. In both cases, the presence of C5b-9(m) during the period of convertase formation induced a dose-dependent inhibition of hemolysis. Under the experimental conditions depicted in Fig. 3, 50% inhibition of formation of the classical- and alternative-pathway C3 convertase was achieved with 25 nM and 15 nM C5b-9(m), respectively.

The above experiments did not distinguish between inhibition by C5b-9(m) of C3 convertase formation and inhibition of the cytolytic development of the convertase sites. That C5b-9(m) inhibited formation of the convertases rather than their C3-cleaving function was indicated by the observation that similar amounts of C3 were cleaved when human C2-deficient serum was incubated with C4b,2a convertase-bearing cells in the presence or absence of the terminal complex (Fig. 3*C*). E^{s}AC14b and E^{s}AC43b cells could be preincubated with C5b-9(m) for 30 min at 30°C and washed, and the inhibitory effect was still observed upon postincubation with C2 or with B and D. These findings suggest that the terminal complex interacts with several of the early-acting components in their cell-bound form. Compatible with this contention was the finding that incubation of E^{s}AC-4b,3b,Bb cells with C5b-9(m) also resulted in a dose-dependent decay of the alternative-pathway convertase sites (data not shown). However, C5b-9(m) did not invoke similar decay of classical C3 convertase sites on E^{s}AC4b,2a cells. The collective results indicate that C5b-9(m) may bind to cell-bound C3b,C4b and C3b,Bb but not to C4b,2a.

C5b-9(m) Inhibits C5 Utilization by C5 Convertase. In addition to inhibiting C3 convertase formation, C5b-9(m) also inhibited cleavage of C5 by the C5 convertase or the binding of C5b to cells. Thus, when cells bearing Ni^{2+}-stabilized classical pathway C5 convertase sites E^{s}AC4b,2a-(C3b)$_n$ were offered purified C5 in the presence or absence of C5b-9(m), addition of terminal complexes to the reaction mixture caused a dose-dependent reduction in hemolysis elicited by C6–C9 (Fig. 4).

C5b-9(m) Does Not Inhibit Lysis of E^{s}AC5b-7 Cells by C8 and C9. To determine whether C5b-9(m) could act at a stage later than C5 cleavage, we also prepared E^{s}AC5b-7 cells and incubated them with 4% EDTA/NHS in the presence or absence of 50 nM C5b-9(m). In this case, however, no

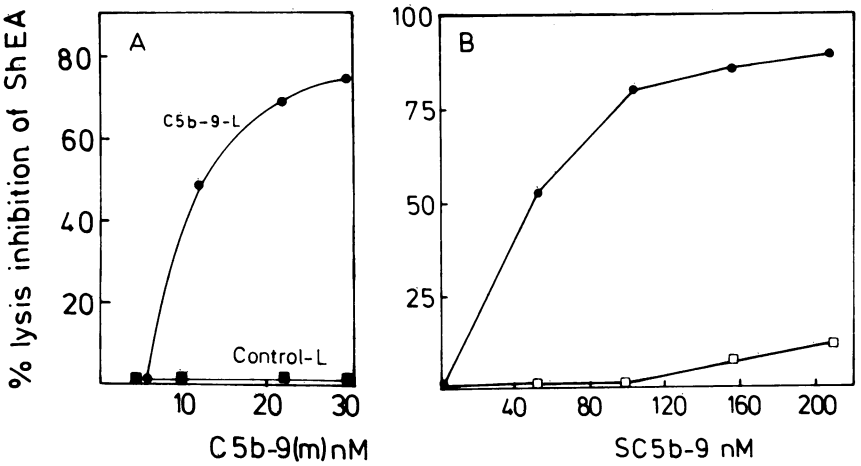

FIG. 2. (*A*) Inhibition of E^{s}A (Sh EA) lysis by liposomes carrying C5b-9(m). Control liposomes were prepared in the absence of C5b-9(m). In the membrane-bound form, the terminal complex ("C5b-9-L") still suppressed hemolysis of E^{s}A by whole human serum. (*B*) In contrast to the membrane-derived form of C5b-9, fluid-phase SC5b-9 complexes isolated as in ref. 19 failed to inhibit hemolysis of E^{s}A (□). Following trypsinization of SC5b-9 (10 μg of enzyme per ml, 1.3 mg of SC5b-9 per ml, 16 hr, 22°C), inhibitory activity became discernable (●).

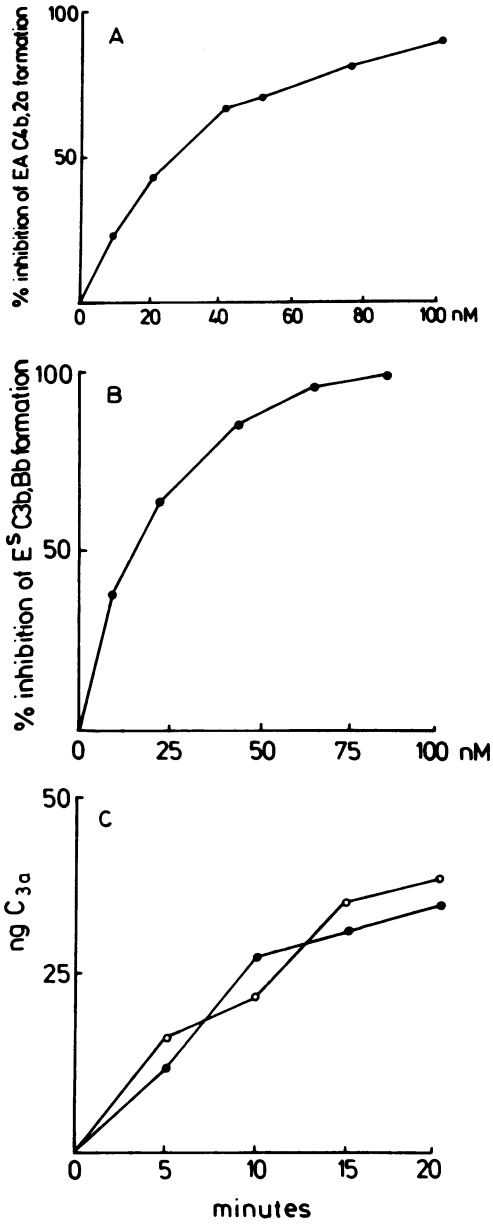

FIG. 3. (*A*) Inhibition of formation of classical-pathway C3 convertase sites. The number of C3 hemolytic sites per cell in the samples incubated with C5b-9(m) was calculated and expressed as percentage of C3 hemolytic sites in the sample in which convertase sites were formed in the absence of C5b-9(m). EA, E^SA. (*B*) Inhibition of formation of alternative-pathway C3 convertase sites. Inhibition of C3 convertase formation was calculated as in *A*. (*C*) Lack of inhibition of C3 cleavage by preformed classical-pathway C3 convertase sites in the presence of C5b-9(m). Cells (2.5×10^8) bearing C4b,2a sites were prepared in $DGVB^{2+}/Ni^{2+}$. After centrifugation and washing, the cells were resuspended in 0.6 ml of a 1:20 dilution of human C2 deficient in $DGVB^{2+}/Ni^{2+}$ serum in the presence (○) or absence (●) of 120 nM C5b-9(m) and incubation was carried out at 37°C. The amount of C3 cleaved was assessed by measuring generation of C3adesArg in the fluid phase. C2-deficient serum was used to avoid generation of new classical-pathway convertase sites on E^SAC4b,2a during the course of the experiment.

inhibition of lysis was observed. Hence, the main action of C5b-9(m) is not on the binding and/or insertion of C8 or C9.

Inhibition of Complement Attack on Autologous Cells and Bacteria. The inhibitory effect of C5b-9(m) was not restricted to heterologous erythrocytes. Similar inhibition of lysis was observed when human erythrocytes sensitized with polyspecific rabbit antibodies were lysed with autologous serum. C5b-9(m) also inhibited killing of the hypersensitive *E. coli* K-12 strain W 3110 by human serum. Under the given experimental conditions (Fig. 5), 2 nM C5b-9(m) elicited 50% inhibition of bacterial killing, and total suppression of bactericidal activity was achieved with 5 nM C5b-9(m).

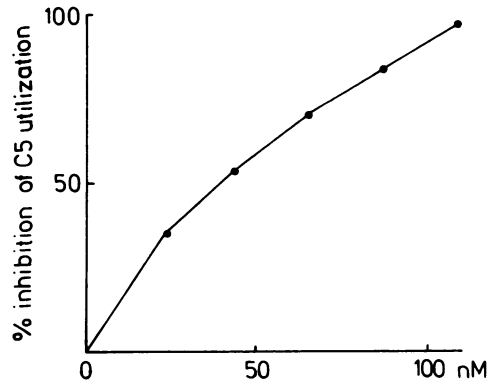

FIG. 4. Inhibition of binding and/or cleavage of C5 by the C5 convertase shown as a function of C5b-9(m) concentration.

DISCUSSION

Three forms of terminal C5b-9 complexes have been defined (16). Two occur naturally *in vivo*: the pore-forming C5b-9(m) complex that is generated on target cell membranes and the SC5b-9 complex that represents its cytolytically inactive counterpart that forms in serum. The latter complex harbors the "S protein" *alias* vitronectin (32, 33), which inactivates C5b-9 by binding to its apolar surfaces. A third complex, designated SC5b-9(m), can be generated *in vitro* by binding of S protein to isolated C5b-9(m) after removal of detergent (16). During attempts to deplete serum of S protein by complexing it to C5b-9(m), we observed that serum receiving purified C5b-9(m) displayed diminished hemolytic and bactericidal titers. Subsequent experiments disclosed that the effect was specifically evoked by C5b-9(m) and not when SC5b-9 was added to serum. This finding emphasizes the fact that SC5b-9 and SC5b-9(m) are conformationally distinct from each other, as noted previously (16).

SC5b-9(m) represents an *in vitro* artefact, and it was consequently of prime importance to determine whether

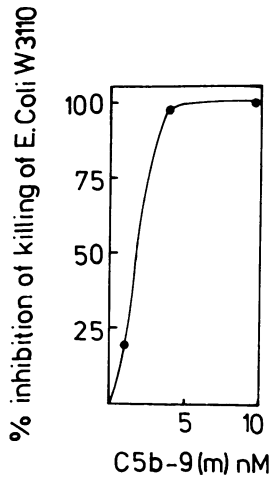

FIG. 5. The serum-sensitive *E. coli* K-12 strain W 3110 was incubated with 2% whole human serum in the presence of C5b-9(m) for 20 min at 37°C. Bactericidal activity was totally suppressed by 5 nM C5b-9(m).

membrane-bound C5b-9(m) was also endowed with the same capacity to inhibit complement activation and E^sA lysis. Affirmative results were obtained when purified C5b-9(m) complexes were incorporated into phosphatidylcholine liposomes, rendering it apparent that feedback inhibition of complement activation will occur whenever the pore-forming complex is generated on membranes. The finding that C5b-7(m) and C5b-8(m) complexes were also inhibitory indicated that the regulatory function must be carried by a molecular region(s) within C5b, C6, or C7. In this context, it is of interest to note that native C5 has earlier been reported to inhibit cleavage of C3 by cell-bound C3 convertase (34).

To determine the stages of the complement sequence at which C5b-9(m)-dependent inhibition occurs, we examined the effect of C5b-9(m) on the formation and the function of C3 convertases and C5 utilization by preformed C5 convertase sites and analyzed possible inhibitory processes occurring after C5b-7(m) formation. We obtained evidence that C5b-9(m) suppressed the formation of the classical- and alternative-pathway C3 convertases in a dose-dependent manner and that it also inhibited utilization of C5 by cell-bound C5 convertase. We have not yet determined whether the latter effect is due to inhibition of C5 cleavage or to suppression of C5b-C9 utilization. C5b-9(m) did not inhibit cleavage of C3 by preformed C3 convertase, and it was also unable to effectively inhibit the lysis of cells laden with C5b-7(m) by C8 and C9. Initial experiments indicated that C5b-9(m) may interact with cell-bound C3b, C4b, and C3bBb but not with fluid-phase complement components.

The present data reveal an unexpected regulatory function of C5b-9(m). It appears meaningful that SC5b-9, whose formation reflects cytolytically ineffective complement activation, is not endowed with this property. Since trypsinization of SC5b-9 partially unmasks its inhibitory capacity, S protein may bind to the domain in the molecule that is required for the inhibitory function. Two considerations together exclude that the described effects were due to the presence of a contaminating membrane regulatory protein derived from the target erythrocytes. (*i*) C5b-9(m) purified from heterologous sheep or rabbit cells was effective in regulating the human activation sequence. Membrane regulatory proteins are generally ineffective in heterologous systems. (*ii*) Regulatory capacity was demasked by trypsinization of SC5b-9, which was prepared from serum in the absence of target membranes.

Feedback inhibition by C5b-9(m) also occurs during complement attack on autologous cells and on bacteria, and it thus seems probable that this regulatory process is generally operative at sites of generation of the cytolytic pathway *in vivo*. As a result, overall complement consumption will be reduced, less C5b-9(m) will be formed, and all complement-dependent processes contributing to the local inflammatory reaction will be diminished. A unique feature of the regulatory process is that inhibition is elicited by a product of the activation sequence and not by a protein that is primarily present in plasma or on the cell membrane.

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