Effect of agents that produce membrane disorder on lysis of erythrocytes by complement

(complement channels/membrane channels/complement cytotoxicity)

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ABSTRACT To evaluate the effect of membrane lipid acyl-chain packing on the efficiency of cell lysis by complement, we have studied membrane modulation by 2-(2-methoxy)-ethoxyethyl-8-(*cis*-2-*n*-octylcyclopropyl)-octanoate (A2C) and by myristoleyl alcohol, the *cis* isomer of a C14:1 aliphatic alcohol. These substances are known to increase the membrane lipid disorder by virtue of the bend in their acyl chains, which is believed to loosen the phospholipid acyl-chain packing. We have found that both of these compounds markedly enhance the lysis of erythrocytes by the terminal complement proteins C5b-9. The enhancing effect by A2C is operative in the formation of erythrocytes carrying complement components C5b, C6, and C7, as well as in the subsequent reactions with complement components C8 and C9. We have also found that A2C-treated erythrocytes bind C5b6 to a measurable extent, whereas untreated erythrocytes do not. We attribute this to a shift in the partition equilibrium of C5b6 toward membrane association, which would improve lytic efficiency. The increase of membrane lipid disorder by these agents would also be expected to increase insertion of hydrophobic peptides from C7, C8, and C9, with consequent gain in lytic efficiency. Treatment of erythrocytes with sublytic doses of NaDodSO₄ or Triton X-100 did not enhance lysis by C5b-9 appreciably, suggesting that enhancement of lysis by C5b-9 is not a general property of amphiphiles.

Membrane attack by complement (C) is initiated when the component C5 is cleaved into C5a and C5b by the C5 convertases of either the classical or alternative activation pathways. The subsequent interactions among the terminal complement proteins (C5b-C9) are accompanied by exposure of hydrophobic peptides (1-3). If this occurs in the immediate vicinity of a bilayer membrane, some of the exposed peptides became inserted in the lipid bilayer (4-6) and are assembled into channels that permit ion flow across the membrane (7-11). In the case of cells that are susceptible to colloid-osmotic disruption, this leads to cytolysis. The processes of insertion and channel formation are affected by the chemical properties of the membrane (12-16). Notably, increasing the cholesterol concentration of the membrane decreases the efficiency of complement attack (12).

We have studied agents that increase membrane disorder with respect to their effect on complement attack. Specifically, we have investigated the action of 2-(2-methoxy)-ethoxyethyl-8-(*cis*-2-*n*-octylcyclopropyl)-octanoate (A2C) and myristoleyl alcohol. A2C is an amphipathic molecule that has a polar domain sufficiently long to bridge the water and hydrocarbon region of the lipid bilayer and a nonpolar part containing a *cis*-substituted cyclopropane ring that promotes disorder within the hydrocarbon region of the membrane (17). Because of partial structural similarity of its hydrocarbon chain, a similar effect is produced by myristoleyl alcohol (18).

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MATERIALS AND METHODS

Complement Components. C5b6 was prepared from human serum according to the method of Yamamoto *et al.* (19). Three different preparations of C5b6 were used. Their titers varied widely and, therefore, their relative concentrations (i.e., reciprocal dilutions) do not reflect comparable hemolytic activities. Guinea pig C7, C8, and C9 were isolated by Carl Hammer as described (5, 20). Hemolytic assays were performed by using 2.5 mM sodium barbital/71 mM NaCl/2.5% dextrose/0.1% gelatin/0.15 mM CaCl₂/1.0 mM MgCl₂, pH 7.4 (BDG²⁺ buffer), as diluting buffer or suspension medium.

Chemicals. A2C (Makor, Jerusalem, Israel) was dispersed in 5 mM sodium barbital/142 mM sodium chloride, pH 7.4 (NaCl/B). This dispersion was sonicated in a bath sonicator at room temperature until a homogeneous turbid colloidal suspension was obtained ($\approx$ 3-5 min). Initially, 2 μ l of A2C was dispersed in 5 ml of the NaCl/B to make a 0.1% stock suspension.

Myristoleyl alcohol (Sigma) or its *trans* isomer (Nu Check Prep, Elysian, MN) (an unsaturated bond at C7-C8 position) were dissolved in chloroform to a concentration of 1%. This solution was mixed with NaCl/B and sonicated with a 1-cm diameter probe at a 40% power setting (Biosonick IV) for 3-5 min at 0°C.

Triton X-100 (Sigma) or NaDodSO₄ (BDH, Poole, England) were dissolved initially in NaCl/B to final concentrations of 0.1 and 0.05 mM, and further dilutions were later made to determine the sublytic concentrations of these detergents for erythrocytes. The binding of these detergents to erythrocyte membranes is not reduced by washing, as judged by measurement of osmotic fragility.

RESULTS

Effect of A2C on Lysis of Sheep Erythrocytes by C5b-9. Equal volumes of erythrocytes (3×10^8 per ml in NaCl/B) and A2C dispersion of the desired concentration were incubated for 4 hr at 37°C. The cells were washed and suspended to 1.5×10^8 per ml in BDG²⁺ buffer. Hemolytic assays were performed with limiting concentrations of human C5b6. Briefly, 0.1 ml of erythrocytes was treated with 0.2 ml of C5b6 at 27°C for 15 min, excess guinea pig C7 (0.1 ml) was added, and the mixture was incubated for 20 min at 27°C. Excess guinea pig C8 and C9 (0.1 ml) were added, and this mixture was incubated for 45 min at 37°C. The results (Fig. 1) indicate a very large increase in lytic efficiency when erythrocytes are modified with A2C. Further-

Abbreviations: A2C, 2-(2-methoxy)-ethoxyethyl-8-(*cis*-2-*n*-octylcyclopropyl)-octanoate; C, complement (individual C components are designated by number—i.e., C8, C9, etc.; the letter b, as in C5b, refers to a fragment of a complement molecule; C5b6 represents a stable complex of C5b and C6); EC5b6,7, erythrocytes carrying C proteins C5b, C6, and C7; NaCl/B, barbital-buffered saline.

FIG. 1. Dose response studies of C5b6 on erythrocytes treated with various concentrations of A2C. Erythrocytes treated with A2C at 0.01% (Δ), 0.025% ($\square$), and 0.05% ($\circ$) for 4 hr were incubated with limited amounts of C5b6 for 15 min at 27°C and then with excess C7, C8 and C9.

more, at each concentration of A2C, the C5b6 dose-response curve was linear.

Effect of A2C on Lysis of Erythrocytes Carrying C5b, C6, and C7 (EC5b6,7) by C8 and C9. To determine which stage or stages of C5b-9-mediated hemolysis was affected by A2C, comparative titrations were performed with erythrocytes and with the cell intermediate EC5b6,7 with and without A2C modulation.

EC5b6,7 were prepared by incubating erythrocytes with various concentrations of C5b6 for 15 min at 27°C and then with excess C7 for 20 min at 27°C. Each of these cell populations was then treated with 0.05% A2C for 1.25 hr at 37°C. After washing, the degree of lysis was evaluated by treatment with excess C8 and C9 for 45 min at 37°C.

The results (Fig. 2) indicate that EC5b6,7 modified with A2C are lysed four times more efficiently than unmodified EC5b6,7.

FIG. 2. Effect of A2C on the reactions of C8 and C9 with EC5b6,7. EC5b6,7 were incubated with 0.05% A2C for 75 min at 37°C; the lytic susceptibility was then compared with that of untreated EC5b6,7. Excess C8 and C9 was used. (Right) EC5b6,7 having incorporated A2C were about 4 times more sensitive than untreated EC5b6,7. (Left) Control—A2C-treated erythrocytes vs. untreated erythrocytes.

FIG. 3. Uptake of C5b6 by erythrocytes treated with A2C. Erythrocytes treated with 0.05% A2C for 75 min at 37°C were incubated with various concentrations of C5b6 for 20 min at 27°C. The cells were centrifuged, washed once with BDG²⁺ buffer, and incubated with C7 for 20 min at 27°C and then with C8 and C9 for 45 min at 37°C. A2C-treated erythrocytes (Δ) were lysed by this treatment; control erythrocytes ($\circ$) showed no lysis.

In the case of erythrocytes, modification with A2C increased lytic susceptibility 25-fold. Thus, the enhancement of lysis by A2C appears to be operative in the formation of EC5b6,7, as well as in the C8 and C9 reactions with EC5b6,7.

Effect of A2C Modulation on the Capacity of Erythrocytes to Bind C5b6. These experiments were designed to determine how well A2C-treated erythrocytes bind C5b6. A2C was incorporated into erythrocytes. After washing, the cells were incubated with appropriate dilutions of C5b6 for 20 min at 27°C. After another washing with BDG²⁺ buffer, the lytic susceptibility was evaluated by incubating the cells with excess C7 for 20 min at 27°C and then with excess C8 and C9 for 45 min at 37°C. Control erythrocytes were treated similarly except for omission of A2C.

The results (Fig. 3) show that cells that had been treated with A2C lysed to a significant extent, whereas the untreated erythrocytes showed no lysis. Thus, the single washing did not remove all of the C5b6 from the A2C-treated cells, suggesting that

FIG. 4. Uptake of C5b6 by erythrocytes treated with various concentrations of A2C. Erythrocytes treated for 75 min with various concentrations of A2C were incubated with C5b6, washed once, and lysed with C7, C8, and C9 as described in legend to Fig. 3. The C5b6 uptake, as judged by lytic response, varied linearly with A2C concentration.

FIG. 5. Lytic susceptibility of erythrocytes treated with myristoleyl alcohol (C14:1) or with its *trans* isomer. Erythrocytes were incubated with *cis* (Δ) or *trans* ($\circ$) alcohol at various concentrations for 2 hr at 37°C, washed with NaCl/B, and then treated with C5b-9. The *cis* alcohol produced much greater enhancement of lysis than the *trans* isomer.

this membrane-modulating reagent improves the binding of C5b6 to erythrocytes.

Fig. 4 shows a similar experiment in which a dose-response curve of A2C was obtained with erythrocytes and a constant dose of C5b6. Again, unmodified erythrocytes did not lyse at all. In other experiments (not shown), it was observed that two washings of A2C-treated EC5b6 reduced their lytic susceptibility markedly, which suggests that binding of C5b6 by A2C-treated erythrocytes, though stronger than that by plain erythrocytes, is reversible.

Effect of *cis* and *trans* C14:1 Alcohols on Lysis of Erythrocytes by C5b-9. Like A2C (17, 21, 22), *cis* unsaturated aliphatic alcohols promote membrane disorder (18, 23, 24). Accordingly, a comparison was made between the capacity of the *cis* and *trans* isomers of C14:1 alcohols to affect lysis by C5b-9. Equal volumes of erythrocytes (3×10^8 per ml of NaCl/B) and alcohol dispersions at the desired concentrations were incubated for 2 hr at 37°C. Under these conditions, near complete uptake of

FIG. 6. Effect of chloroform treatment on lysis of erythrocytes by C5b-9. Erythrocytes were incubated in the presence (Δ) and absence ($\circ$) of a sonicated 1/2000 dispersion of chloroform in NaCl/B for 2 hr at 37°C. The cells were washed and lysed with various concentrations of C5b6 and excess C7, C8, and C9.

the alcohols into the erythrocyte membrane is expected because the partition coefficients of these long chain alcohols are extremely high (24). Accordingly, when erythrocytes were treated with supernatants of the initial incubation, no lytic enhancement by C5b-9 was observed. The cells that were treated with alcohols were washed, and hemolytic assays were performed as in the case of A2C-treated erythrocytes.

The results show that the lytic enhancement by the *cis* isomer was much greater than that by the *trans* isomer (Fig. 5), as would be expected from their respective membrane-disordering capacities (24). Because the initial dispersion of the alcohols contained a small quantity of chloroform (1/2000), a control experiment with a similar dispersion of chloroform in buffer was performed. Under those conditions, chloroform had no measurable effect on lysis of erythrocytes by C5b-9 (Fig. 6).

Effect of Triton X-100 or NaDodSO₄ on Lysis of Erythrocytes by C5b-9. A2C is an amphiphilic substance that lyses erythrocytes when used at concentrations $>0.1\%$. Hence, it was deemed of interest to study the effects of other amphiphiles, such as Triton X-100 and NaDodSO₄, on lysis of erythrocytes by C5b-9. As the highest concentration of A2C (0.05%) used in the present experiments lies just below the lytic threshold, it was necessary to determine this threshold for these amphiphiles. Accordingly, hemolytic dose-response experiments were performed with Triton X-100 and NaDodSO₄. Measurements of the hemolytic efficiency of C5b-9 were then performed with erythrocytes that had been treated with these detergents at concentrations just below the lytic thresholds, as well as at concentrations that lysed 5–7% of the erythrocytes. The results suggest that NaDodSO₄ had essentially no effect while the lysis of Triton-treated erythrocytes by C5b-9 was only slightly enhanced (Fig. 7). In this context, it is also of interest that 1/2000 dispersion of chloroform in buffer, a sublytic dose, had no effect on the lytic activity of C5b-9 (see Fig. 6).

DISCUSSION

The susceptibility of cells to attack by C5b-9 has been shown to be influenced by agents or conditions that affect the membrane surface, such as polycations (25), the ionic strength of the reaction medium (26), or neuraminidase treatment (27), all of which are believed to modify the interaction of the membrane surface with complement proteins or inhibitors. A second way in which susceptibility to C5b-9 can be influenced involves the

FIG. 7. Effect of NaDodSO₄ or Triton X-100 treatment of erythrocytes on lysis by C5b-9. Erythrocytes treated with NaDodSO₄ (Left) or Triton X-100 (Right) at sublytic concentrations [50 μ M ($\blacktriangle$) and 31.25 μ M ($\triangle$), respectively] and at concentrations that lysed 5–7% of the erythrocytes [100 μ M ($\blacksquare$) and 62.5 μ M ($\square$), respectively] and were washed once and assayed with C5b-9. Control ($\bullet$ and $\circ$). Experiments with NaDodSO₄ and with Triton X-100 were performed at different times with different erythrocyte populations.

use of factors that modify the lipid bilayer proper. Our work on membrane modulation has been addressed to this aspect (12, 16, 28).

It has been shown that incorporation of A2C into membranes produces disorder in the bilayer packing (17, 21, 22, 24). This effect is believed to be similar to that caused by *cis* unsaturated acyl chains. The promotion of disorder has been attributed to the *cis* cyclopropane ring in A2C and the *cis* unsaturated bond in acyl chains (17, 29, 30). Kosower *et al.* (17) selected A2C as the most effective membrane-mobility agent from a group of homologous compounds. An important advantage of A2C, as contrasted with unsaturated acyl chains, is its insusceptibility to oxidation.

Alteration of membranes by A2C results in changes of their biological behavior, such as enhancement of lymphocyte cap formation, and increase in erythrocyte membrane permeability to the reduced glutathione oxidant 1,2-diazenedicarboxylic acid bis(*N'*-methylpiperazide) (21, 22). Also, altered distribution of human erythrocytes in a two-phase dextran/polyethylene glycol/water system has been reported (22). This is thought to be due to creation of "defects" in the lipid bilayer.

We now show that incorporation of A2C into the erythrocyte membrane markedly enhances lysis by C5b-9. The enhancing effect of A2C is operative both in the formation of EC5b6,7 and in the subsequent reactions with C8 and C9 (see Fig. 2). It has also been found that A2C-treated erythrocytes bind C5b6 more tightly than untreated erythrocytes (see Fig. 3). This effect would be expected to promote the efficiency of EC5b6,7 formation.

We have also found that myristoleyl alcohol, the *cis* isomer of a C14:1 alcohol, enhances the hemolytic efficiency of C5b-9 markedly. As expected, the *trans* isomer had a smaller enhancing effect than the *cis* isomer (see Fig. 5). Comparative experiments with the detergents Triton X-100 and NaDodSO₄ showed little or no enhancement when used at concentrations just below or above the lytic threshold (see Fig. 7). Therefore, enhancement of lysis by C5b-9 is not a general property of amphiphilic substances.

To interpret the action of A2C on hemolysis of erythrocytes by C5b-9, it is helpful to consider its effects of EC5b6 and EC5b-9 formation separately because C5b6 differs markedly from C5b-7, C5b-8, and C5b-9 with respect to stability in the aqueous phase and to affinity for lipid bilayers. Because C5b6 does not decay in the aqueous phase, its reaction with lipid bilayer membranes can be considered as a process of partition of a solute between two immiscible liquids. As shown in Figs. 3 and 4, infiltration of the membrane with A2C shifts the partition of C5b6 toward the membrane. The greater uptake of C5b6 by the membrane, in turn, should promote the subsequent reactions involving C7, C8, and C9. This effect is similar to that produced by membrane-bound C3b (31); however, the mechanism by which A2C shifts the equilibrium distribution may not be the same as that in the case of membrane-bound C3b.

When C5b6 reacts with C7-C9 in the aqueous phase, the resulting C5b-7, C5b-8, and C5b-9 complexes lose the capacity to attack membranes, unlike C5b6, which is stable in aqueous medium. Furthermore, when these complexes are formed in a membrane, the binding is irreversible, whereas C5b6 associates only loosely with membranes. For these reasons, the reactions of C7, C8, and C9 with membranes carrying C5b6 have to be considered as competitive processes between insertion of hydrophobic peptides from C7, C8, and C9 and inactivation of these hydrophobic peptides in the aqueous phase. On the basis of these considerations, substances such as A2C or myristoleyl alcohol, which are believed to increase membrane lipid disorder,

would be expected to enhance hemolytic efficiency by promoting the interaction between the hydrophobic complement peptides and the membrane.

These considerations may be applicable to other substances that can produce membrane disorder. This includes unsaturated phospholipid acyl chains that are naturally present in membranes. In studies of the effect of membrane modulation on complement attack, A2C is a valuable reagent because of its high degree of effectiveness and its resistance to oxidation.

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